FACTORS INFLUENCING NATURAL ATTENUATION OF DINITROTOLUENES IN SURFACE SOILS: BADGER ARMY AMMUNITION PLANT A CASE STUDY

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FACTORS INFLUENCING NATURAL ATTENUATION OF DINITROTOLUENES IN SURFACE SOILS: BADGER ARMY AMMUNITION PLANT A CASE STUDY

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To Dinesh, my dear Husband

and my best Friend

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Chemical Compounds

ACN	Acetonitrile
DNT	Dinitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
NAC	Nitroaromatic Compound
TNT	Trinitrotoluene

Biological and Engineering Notation

X	Biomass Concentration (μM)
S	Substrate Concentration (μM)
K_s	Half-Velocity Constant (μmol S/L)
k	Maximum Substrate Utilization Rate (d^{-1})
Y	Yield Coefficient (μmol S/μmol X·d)
b	Bacterial Decay Coefficient (d^{-1})
K_d	Soil-Water Partition Coefficient (L/kg soil)
K_{ow}	Octagonal-Water Partition Coefficient
K_{oc}	Organic Carbon Partition Coefficient
f_{oc}	Fraction of Organic Carbon (wt/wt)
CSTR	Continuously Stirred Tank Reactor
HRT	Hydraulic Retention Time (d^{-1})
OC	Organic Carbon
TOC	Total Organic Carbon

Other

BAAP	Badger Army Ammunition Plant
HPLC	High-Performance Liquid Chromatography
IC	Ion Chromatography
MNA	Monitored Natural Attenuation
OP1	Settling Pond 1
OP4	Settling Pond 4
SSB	Spoils Disposal Area
AT123D	Analytical Transient 1-, 2-, and 3- Dimensional
SESOIL	Seasonal Soil

SUMMARY

The purpose of this research was to evaluate factors influencing natural attenuation of dinitrotoluenes (DNTs) in surface soils. The impetus for the work arose from recent investigations on the residual DNT in the Settling Ponds and Spoils Disposal Areas at the Badger Army Ammunition Plant (BAAP). BAAP was constructed in 1942 to produce propellants. Production of explosives was terminated in 1975, and remedial investigation and activities associated with contaminated soil and groundwater started in 1988. Current work focuses on assessment of factors affecting natural attenuation of DNT and the application of monitored natural attenuation (MNA) as a remediation strategy for the residual DNT remaining at the site. Based on the previous research involving contaminated media obtained from locations at BAAP, and the fact that groundwater at the site is not contaminated, it seemed likely that aerobic biodegradation of DNT is active without intervention, and that natural attenuation may be an effective strategy for managing the contamination that exists at BAAP.

The first objective of this study was designed to determine whether or not microorganisms capable of growth on DNT in the soils and subsurface materials are present at the Settling Ponds and Spoils Disposal Areas. Microcosms constructed with BAAP soils showed that microbes indigenous to soils are capable of 2,4-DNT mineralization, but not 2,6-DNT.

The second objective of this work was to evaluate factors that would influence the rate and extent of natural attenuation of DNTs in the vadose zone soils. To serve these purpose three different experiments were carried out:

1. Batch Sorption Study (to evaluate bioavailability)
2. Soil Column Study (to evaluate nutrient availability)
3. Chemostat Study (to study the effect of low temperatures on rate and extent of DNT degradation).

Batch adsorption test were carried out to determine sorption characteristics and hence the bioavailability of DNT, which were best predicted with the Freundlich isotherm. Sorption tests were followed by a batch desorption test to assess contaminant release. In batch sorption experiments 2,4- and 2,6-DNT behaved similarly for all but one test, but K_F values varied greatly depending on the soil sample. The sorption studies showed that DNT will adsorb reversibly and become bioavailable, desorbing at different rates depending presumably, on soil properties.

Column studies were carried out in two glass columns containing soils from Settling Pond 1 (OP1) (soil bed, 10 cm) to assess leaching and biodegradation under simulated infiltration events. One column was fed 2,4-DNT alone and the other was fed 2,6-DNT only. Simulated rain water (10 mL) containing DNT (10 mg/L) was applied everyday. The effluent collected was analyzed for DNT, nitrite and nitrate. 2,4-DNT was not detected throughout 123 days of operation in effluent samples. Nitrite (3- 5 μM) was detected but at less than stoichiometric amounts (85 μM), presumably due to oxidization by nitrite oxidizers. Breakthrough of 2,6-DNT occurred from the column after 22 days and reached steady state near 41 days. Nitrite was never observed in effluent but 2-methyl-3-nitroaniline (a reduced form of 2,6-DNT) was observed. These findings, coupled with the confirmation of presence of nitrite oxidizers and weak association of DNT with soil OP1 suggest that biodegradation was responsible for removal of 2,4-DNT

in the soil column study. The use of simulated rainwater as influent with no nutrient amendments suggests that nutrients do not limit the biodegradation of low concentrations of DNT in the soil.

Seasonal fluctuation in temperature of surface soils is a concern for monitored natural attenuation at this particular site. At BAAP the temperature varies from -17°C to 32°C which led us to evaluate the effect of variation in temperature on the biodegradation of DNT. A chemostat study was carried out at hydraulic retention time (HRT) of 2.5 days. The temperature was decreased stepwise (22°C, 15°C, 10°C, 7.5°C and, 4°C). The chemostat was monitored for DNT and nitrite. No sustained change in the substrate removal was observed with change in temperature. This suggests that the seasonal fluctuations in temperature will have minimal effect on the DNT removal via biodegradation at temperatures above 0°C.

The effluent samples from the chemostat were also analyzed for nitrite. The nitrite concentrations 22°C were detected to be lower than the expected stoichiometric concentration. The presence of nitrite oxidizing bacteria were confirmed in the mixed culture from soil OP1, used for this experiment and were believed to account for the lower concentrations of nitrite at this temperature. Even though the lowering of temperature did not have any effect on 2,4-DNT degrading bacteria, it had an large effect on the nitrite oxidizers. The activity of nitrite oxidizers decreased with lowering the temperature from 22°C to 15°C which was evident by increase in effluent nitrite concentrations detected at 15°C than that observed at 22°C. Further decreasing the temperature to 10°C and lower ceased the nitrite oxidation and stoichiometric release of nitrite was observed. Thus, it should be noted that while monitoring natural attenuation

during different seasons, none or lower nitrite concentrations at higher temperatures does not indicate none or lower DNT degradation. Similarly higher nitrite concentrations at lower temperature compared to nitrite concentrations observed at higher temperatures does not indicate faster or higher DNT degradation but might suggest lower or no activity of nitrite oxidizers. Thus the fluctuation in nitrite measurement does not necessarily indicate fluctuation in DNT degradation.

Thus it could be concluded from this work that 2,4-DNT degrading bacteria are present in the contaminated areas of the site. Bioavailability of DNT, nutrient limitations, and fluctuation in temperature will not limit the biodegradation of DNT. Nitrite is generally taken as a line of evidence for biodegradation of DNT. The results from the soil column study and chemostat showed that nitrite measurement should not be always taken as a conclusive indicator of DNT degradation. It should be taken into consideration that no nitrite observed could be a false negative that no DNT degradation is occurring at the site (probably at high temperatures).

CHAPTER 1

INTRODUCTION

Dinitrotoluenes (DNT) are produced during the synthesis of the explosive 2,4,6-trinitrotoluene (TNT) via step nitration process. Also, they are precursors of toluene diisocynate used in the production of polyurethane foams [1]. During DNT synthesis the most common isomers produced are 2,4-DNT and 2,6-DNT in the molar ratio of 4:1 [2]. Improper disposal and handling practices associated with manufacturing of TNT have led to contamination of soils and groundwater at army ammunition plants and production sites. TNT production was terminated twenty years ago, but 2,4-DNT and 2,6-DNT remain common contaminants of TNT manufacturing facilities [3]. Both isomers are classified as priority pollutants by the U.S EPA because they exhibit acute toxicity and low level carcinogenicity [4, 5]. EPA treatment standards are 140 mg/kg in soil (0.32 mg/L in water) for 2,4-DNT and 28 mg/kg in soil (0.55 mg/L in water) for 2,6-DNT (40 CFR, Section 268.48). Because of the hazards DNT pose for drinking water supplies, remediation of the contaminated sites is required.

Extensive research has been conducted on the microbial transformations, of nitroaromatic compounds [6, 7] under both anaerobic and aerobic conditions Under anaerobic conditions DNT is cometabolized and mineralization does not occur [8-11]. Under aerobic conditions complete mineralization of DNT occurs and DNT serves as sole carbon, nitrogen and energy source for the growth of the DNT degrading microorganisms [8, 12, 13]. Studies has been conducted to determine the potential of bioremediation of nitroaromatic compounds as a treatment option and its application to clean contaminated sites [14]. Various ex-situ techniques have been applied to remove DNT contamination

from soil and groundwater [12, 15-19], but less focus have been given to *in situ* processes (natural attenuation) for dealing with the contaminated sites.

An alternative to aggressive bioremediation is natural attenuation which refers to the stabilization or destruction of contaminants *in situ* by physical, chemical, or biological means without human intervention. To confirm that destruction of contaminants is occurring, the U.S. Environmental Protection Agency recommends that field or microcosm studies are conducted with contaminated site media to demonstrate biological degradation processes which can be taken as a line of site-specific evidence to suggests that "monitored natural attenuation" (MNA) is an effective site remediation alternative [20].

There are a number of factors that are required to be taken into consideration for natural attenuation to be a viable treatment option at a contaminated site:

1. Whether biodegradation of the contaminant has ever been reported (the answer is affirmative for DNT).
2. The presence of appropriate degrading organisms at the contaminated site.
3. Favorable environmental conditions to sustain the activity of the microorganisms through the completion of MNA:
    I. The fate and bioavailability of contaminants in the subsurface
    II. Availability of oxygen (water content)
    III. Availability of nutrients
    IV. pH
    V. Temperature

VI. Toxicity of or inhibition by the concentration of substrate or the byproducts to the microbes involved in the degradation.

Fortner *et. al.* [21] studied factors affecting the *in situ* biodegradation of 2,4-DNT in highly contaminated soils. They employed soil columns and respirometer studies to analyze the effects of availability of oxygen, nutrients limitations, variation of pH and concentration of nitrite (a byproduct) on the degradation of 2,4-DNT in the contaminated soils. The test soils used by Fortner *et. al.*, were obtained from Badger Army Ammunition Plant (BAAP). The soil column studies conducted at field capacity demonstrated that high levels of 2,4-DNT can be biodegraded by addition of complete mineral medium [22] but not by bicarbonate–buffered distilled or deionized water or by phosphate–amended tap water. The respirometer studies demonstrated that the high levels of nitrite inhibits further degradation and that at pH below 6.0, 2,4-DNT degradation slows rapidly [21].

The focus of the current research is to assess "monitored natural attenuation" (MNA) as an effective alternative for remediation of soils with low concentration of DNT contamination. The soils used in this study were obtained from Badger Army Ammunition Plant (BAAP) in Baraboo, Wisconsin, which have been contaminated for more than 30 years. The overarching objective of this study is to assess the role of naturally occurring biodegradation in the natural attenuation of DNT contamination in surface soils and the underlying vadose zone with application to BAAP field conditions. The specific objectives of the proposed work are:

1. Confirm the presence and activity of DNT degrading bacteria in samples taken from contaminated soils and underlying vadose zone materials at

BAAP. Based on previous studies it is hypothesized that organisms capable of degrading DNT are present at the site [15, 19, 21, 23].

2. Evaluate the influence of bioavailability of DNT, nutrients limitation and, fluctuation in temperature on the rate and extent of natural attenuation of DNTs in vadose zone soils under simulated field conditions including rainwater infiltration, transport and, degradation. It is hypothesized that since concentrations of DNT are low (1.9–470 mg/kg in Spoils Disposal Areas and 0.64–110 mg/kg in Settling Ponds for 2,4-DNT and 0.11– 32 mg/kg in Spoils Disposal Areas and ND–6.80 mg/kg Settling Ponds for 2,6-DNT) availability of oxygen, nutrients, pH, and nitrite inhibition will not limit DNT degradation.
3. Examine the use of nitrite as a line of evidence of DNT biodegradation for monitoring natural attenuation

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

This chapter is organized into two sections: the background information and the literature review of DNT followed by brief background information about nitrification.

2.1. Dinitrotoluenes

The background section gives an overview of the synthesis of 2,4-DNT and 2,6-DNT, the widespread contamination of DNT in the United States, and the metabolic pathways of 2,4-DNT and 2,6-DNT biodegradation. The literature review first describes kinetics and stoichiometry of DNT degradation. It is followed by the description of sorption characteristics of DNT. It is important that the characteristics of these compounds be understood to allow assessment of the fate and transport of DNT at the contaminated sites.

2.1.1. Background

2.1.1.1. Synthesis of DNT

Dinitrotoluenes (DNTs) are precursors of 2,4,6-trinitrotoluene (TNT), once the most widely manufactured military explosive in the world. TNT was produced by the step nitration of toluene as shown in Figure 2.1. The first nitration produces 2-nitrotoluene and 4-nitrotoluene in almost equal amounts. The second nitration step yields 76% 2,4-DNT, 19% 2,6-DNT, and 5% of other isomers. The third nitration produces TNT [8].

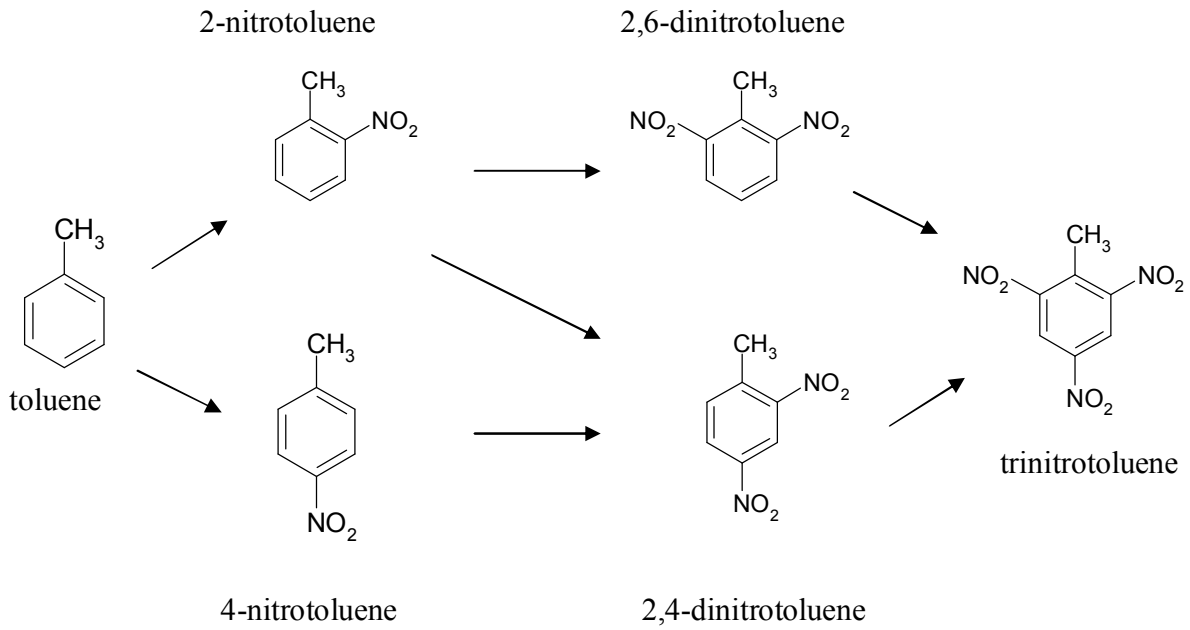


Figure 2.1. TNT production by a three step nitration of toluene [3].

Safety considerations dictated that the reactors for each nitration step be housed in separate buildings and for similar reasons manufacturing facilities were geographically separated and mostly located in remote areas [3]. The production of TNT required large quantities of water for the purification of the product. Therefore, ammunition plants were also often located near large water reservoirs [24]. Due the distributed method of TNT manufacture, improper handling and disposal practices have led to widespread contamination. The major explosives manufacturing, handling, and storage sites are shown in the map (Figure 2.2). The soil samples used in this study were obtained from Badger Army Ammunition Plant (Badger AAP), which is one of the contaminated sites shown in the map. The details of contamination and concerns at the site are discussed in Chapter 3. TNT is no longer produced in U.S., but DNT still remains an important industrial chemical as the precursors of toluene diisocynate used in the production of polyurethane foams. Environmental release of DNT is rare in current industrial processes [8].

2.1.1.2. Aerobic Biodegradation of 2,4-dinitrotoluene and 2,6-dinitrotoluene

Degradation of DNT was initially observed 20 years ago, but only recently the degradation mechanisms have been elucidated and the bacteria have been isolated. Strains that grow on single DNT isomers as sole carbon, nitrogen and energy sources have been isolated from the contaminated systems worldwide [8]. Also, strains that can grow on 2,4-DNT and 2,6- DNT have been isolated from bioreactors receiving mixtures of DNT isomers. 2,4-DNT strains are not capable of growing on 2,6-DNT and vice versa is also true [8].

The 2,4-DNT degradative pathway (Figure 2.3) was determined in *Burkholderia*

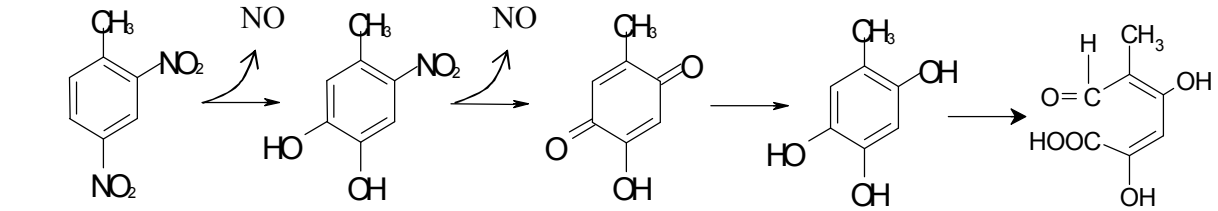
Figure 2.2. Explosive-contaminated manufacturing, processing, and storage sites in the U. S. A, Arsenal; AAP, Army Ammunition Plant; AD, Army Depot; AFB, Air Force Base; ANGF, Air National Guard Field; NSB, Naval Submarine Base; OW, Ordnance Works, and PG, Proving Ground [29].

sp. strain DNT (the first DNT degrading strain to be isolated and studied). An initial dioxygenase attack at the 4-nitro position converts 2,4-DNT to 4-methyl-5-nitrocatechol (4M5NC) with the release of nitrite [22]. A monooxygenase attack at the remaining nitro-substituted position converts 4M4NC to 2-hydroxy-5-methyl-quinone (HMQ) with the release of the second nitro group as nitrite [13]. A quinone reductase converts HMQ to 2,4,5-trihydroxytoluene (THT) in a reaction requiring NADH. The product of THT ring cleavage is unstable and attempts at purification results in decomposition to a compound that is not metabolized by the bacteria [13]. Properties of the purified ring cleavage enzyme and genetic evidence strongly indicates that THT undergoes *meta*-ring cleavage fission catalyzed by an extradiol dioxygenase [25].

The 2,6-DNT degradation pathway (Figure 2.3) is not identical to the 2,4-DNT degradation pathway. The initial dioxygenase attack is similar but the product formed is 3-methly-4-nitrocatechol (3M4NC), with the release of first nitro group as nitrite. 3M4NC undergoes direct meta-ring cleavage catalyzed by a catechol-2,3-dioxygenase to produce 2-hydroxy-5-nitro-6-oxohepta-2-4-dienoic acid. The remaining nitro group is released as nitrite in subsequent reactions that have not been characterized [8].

When both 2,4-DNT and 2,6-DNT are present together at contaminated sites, the presence of one DNT affects the degradation of the other. 2,6-DNT degradation is inhibited by high relative 2,4-DNT concentrations[26]. Conversely, 2,6-DNT concentrations as low as 100 μM inhibits degradation of 2,4-DNT in some strains [27]. Nitrite is toxic to *Burkholderia* sp. strain DNT at concentrations above 10 mM, and other 2,4-DNT- degrading strains are inhibited at nitrite concentrations ≥ 40 mM [21]. 2,6-

(a)



(b)

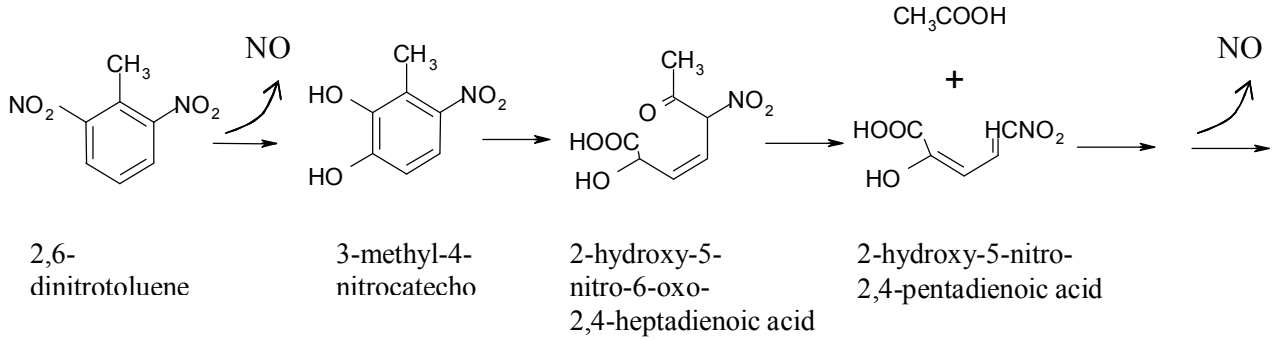


Figure 2.3. Degradation pathways for (a) 2,4-dinitrotoluene and (b) 2,6-dinitrotoluene [2].

DNT-degrading strains are unaffected by nitrite concentrations up to 100 mM. The inhibitory effects of DNT and nitrite on DNT degradation are important considerations in the design of remediation strategies.

Under anaerobic conditions it has been shown that DNT is cometabolized and mineralization does not occur [8-11]. However, cometabolic reduction of the nitro group under both anaerobic and aerobic conditions by non-specific nitroreductases has been reported. Non-specific reduction does not lead to ring cleavage and further transformation of the metabolites [8].

2.1.2. Literature Review

2.1.2.1. Microbial Kinetics and Stoichiometry

Microorganisms grow under favorable physicochemical conditions. In a batch culture, a typical growth curve for a population of cells is illustrated in Figure 2.4. The growth curve can be divided into distinct phases: 1) lag phase, 2) exponential phase, 3) stationary phase and, 4) decay phase.

Lag phase occurs immediately when a microbial population is inoculated into a fresh medium or when the environmental conditions are changed. During lag phase the microorganisms get acclimatized to the new environment. In the exponential phase the cell have acclimatized and are multiplying rapidly. In the stationary phase the essential nutrients are used up or the product formed has built up an inhibitory level which ceases during the exponential phase. During this phase the net growth is zero. Stationary phase is followed by death phase where cell death and decay occurs [28].

The relationship most frequently used to describe microbial growth kinetics is the semi-empirical Monod equation. It describes the rate of substrate (electron donor) utilized

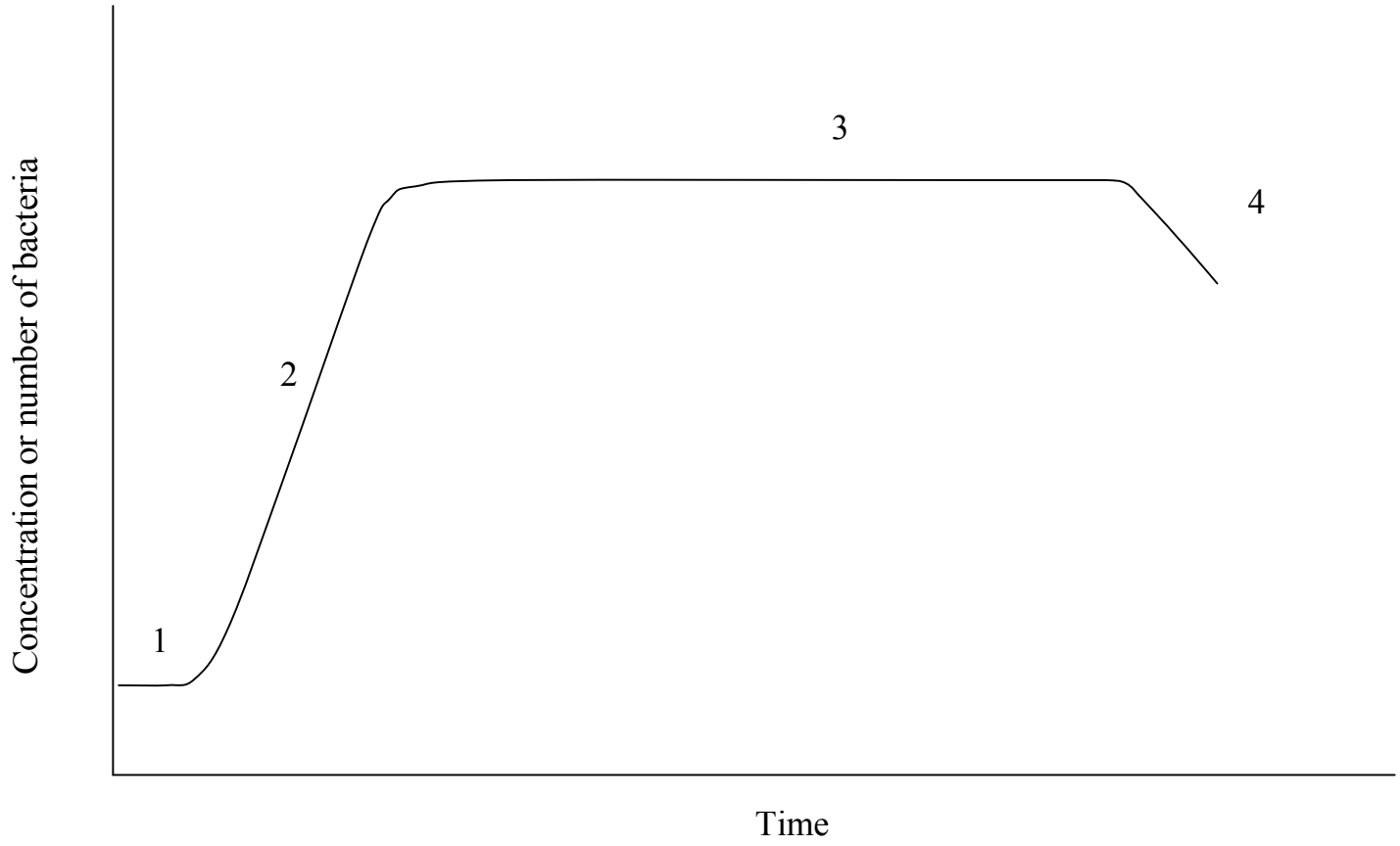


Figure 2.4. Bacterial growth curve with distinct stages of a growth cycle: 1) lag phase 2) exponential phase, 3) stationary phase, 4) decay phase.

as shown in Equation (2.1).

$$\frac{dS}{dt} = -\left(\frac{kXS}{K_s + S}\right) \tag{2.1}$$

The net growth rate of the microorganisms can be represented by the Equation (2.2).

$$\frac{dX}{dt} = Y\left(\frac{kSX}{K_s + S}\right) - bX \tag{2.2}$$

where, S is the rate–limiting substrate concentration (μM), X is the active biomass concentration (μM), t is time (d), K_s is the rate–limiting substrate concentration resulting in one-half the maximum growth rate (μmol S/L), Y is the yield coefficient of bacteria and is the amount of cells produced per amount of substrate utilized (μmol X/ μmol S), b is the endogenous decay rate (d^{-1}) and k is the maximum specific rate of substrate utilization (μmol S/μmol X · d). The biokinetic coefficients are constant but vary with environmental conditions including temperature and pH [29]. The maximum specific rate of substrate utilization (k) is affected by temperature. The value of k roughly doubles for each 10°C increase in temperature for temperatures below the microorganism's optimal growth temperature. This phenomenon can be approximated by Equation (2.3).

$$k = k_{T^R}(1.07)^{(T-T^R)} \tag{2.3}$$

where, T^R is any reference temperature (°C) for which $k_T{}^R$ is known, T (°C) is the temperature at which k value needs to be determined.

Three studies using different approaches have been conducted to determine the biokinetic and stoichiometric coefficients involved in biodegradation of DNT. The first study was carried out by Smets *et. al.* [17], to determine the biokinetic coefficient k and K_s in an aerobic fluidized-bed biofilm reactor using a mechanistic mathematical biofilm

model. The k values for 2,4-DNT were 0.83 to 0.98 g DNT/g X · COD · d and for 2,6-DNT were 0.14 to 0.33 g DNT/g X · COD · d. K_s value for 2,4-DNT were 0.029 to 0.36 g DNT/m^3 and for 2,6-DNT were 0.21 to 0.84 g DNT/m^3 [17].

The second study conducted by Heinz *et. al.* [18] used a batch culture to determine kinetic coefficients. The yield coefficient Y was determined to be 0.3 ± 0.05 g X/ g DNT based on mass balance. The maximum specific growth rate $\mu_{max} = 0.1\ h^{-1}$ and K_s = 0.01 to 0.03 mmol/L were calculated by fitting the experimental data to Monod equation [18].

Daprato *et. al.* [30], used thermodynamics to predict the stoichiometric coefficient based on a method described by Rittmann and McCarty [29]. The method uses the change in Gibbs Free energy (ΔG) of the half reactions involved in DNT degradation for estimating the true yield coefficient Y which was reported as 0.23 g X/g DNT. Based on the yield coefficient the stoichiometric equation for DNT degradation was determined and is shown in Equation (2.4) [30].

$$C_7H_6N_2O_4 + 5.62\ O_2 \rightarrow$$

$$1.63\ NO_2^- + 1.63\ H^+ + 5.17\ CO_2 + 0.4\ C_5H_7NO_2 + 0.90\ H_2O \qquad (2.4)$$

Biodegradation of DNT results in the production of 1.63 moles of nitrite, 5.17 moles of CO_2, 1.63 moles of H_2O, 1.63 moles of H^+ along with biomass growth ($C_5H_7NO_2$). Production of nitrite is generally taken as an indictor of DNT biodegradation.

2.1.2.2. Sorption Properties of 2,4-DNT and 2,6-DNT

The process in which chemicals become associated with solid phases is referred to as sorption. Sorption is important because it may affect the fate and impact of chemicals in the environment. The molecular transfer into the microorganisms is frequently a

prerequisite to a substance's biodegradation. Thus the greater ease of chemical movement from solution versus from solids to bacteria generally causes the biological decomposition of the sorbed form of the chemical to be slower than its dissolved counterpart.

Several conceptual and empirical models have been used to describe equilibrium phase distributions for the sorption of organic compounds in subsurface systems. Linear sorption models is the simplest of these and can be described by a distribution coefficient (K_d) which is a ratio of concentration of chemical on the solid phase (C_s) to the concentration of chemical in the aqueous phase (C_w) at equilibrium.

The thermodynamic justification for this interpretation can be given by relating the distribution coefficient to the activity of the solute in the sorbed and aqueous phase. It is assumed here that in the solutions are sufficiently dilute and thus the activity coefficients of the solute approach limiting values [31, 32]. Sorption of a chemical by solid depends on a number of factors such as, soil properties (% organic matter, cation exchange capacity), solution parameters (pH, ionic strength) and properties of the chemical of interest (K_{ow}). The linear distribution coefficient (K_d) can be written as [33].

$$K_d = \frac{C_s}{C_w} = \frac{C_{oc} f_{oc} + C_{\min} A + C_{ie} s_{ie} A + C_{rxn} s_{rxne} A}{C_{w,neutral} + C_{w,ionic}} \tag{2.5}$$

where,

C_{oc} is the concentration of sorbate associated with the natural organic carbon,
f_{oc} is the weight fraction of solid which is natural organic carbon,
C_{min} is the concentration of the chemical associated with the mineral surface,
A is the area of the mineral surface per mass of solid,
C_{ie} is the concentration of ionized chemical associated with charged sites on the solid surface,
σ_{ie} is the net concentration of charged sites on the solid surface,
C_{rxn} is the concentration of the chemical bonded in a reversible reaction to the solid surface,
σ_{rxn} is the concentration of reactive sites on the solid surface,

$C_{w,neutral}$ is the concentration of the neutral chemical in solution, and
$C_{w,ionic}$ is the concentration of the charged chemical in solution.

Chiou *et. al.* (1979) [34] have indicated that sorption phenomena dominated by partition based processes should be governed by linear isotherms until aqueous-phase solubility is approached, while Karickoff (1981) [31] has suggested that if the aqueous concentration is below 10^{-5} M, or less than one-half the aqueous solubility of the solute, the resulting sorption isotherm will be linear [35].

The energetics of sorption is generally far more complex in practice than implied by the linear model. It is infact unrealistic to expect that models based on a single linear partitioning mechanism or a limiting sorption capacity will be appropriate for most environmentally relevant sorbate-sorbent systems. Thus equilibrium sorption data are often best described by models that can accommodate heterogeneous site energies, the most simple of which is the Freundlich model [35]:

$$C_s = K_F \cdot C_w^{\,n} \tag{2.6}$$

where, K_F is the Freundlich capacity coefficient $(mg/kg)/(mg/L)^n$, Freundlich coefficient describes sorption intensity, values for n less than 1 result in convex adsorption isotherms, while values of n greater than 1 lead to concave adsorption isotherms; the model reduces to the linear form given in Equation (2.5) when n equals 1. Values of K_F and n are generally obtained by fitting log transformed sorption data to the linearized logarithmic form of the Freundlich isotherms as given in Equation (2.7).

$$\log C_s = \log K_F + n \log C_w \tag{2.7}$$

although, more reliable estimates of K_F and n are obtained by a non-linear regression of raw (untransformed) data [35].

The sorption of dinitrotoluenes is governed by two mechanisms. First, being neutral hydrophobic compounds, they sorb to the solid organic carbon component of the soil matrix. Such interactions are weak and nonspecific. Partitioning of hydrophobic organic compounds into particulate organic carbon is often the major sorption mechanism for solid matrices having fraction of organic carbon (i.e. f_{oc}) greater than ($5x10^{-2}$ kg_{oc}/kg_{solid}) [33]. There are models that have been applied successfully to predict the sorption and transport of contaminants when sorption is due to organic carbon. This type of partitioning can be described by organic carbon–water partitioning coefficient K_{oc} which is the ratio of the concentration of chemical in the organic phase (C_{oc}) to the concentration of neutral chemical in the aqueous phase (C_w) at equilibrium and given in Equation (2.8) [33].

$$K_{oc} = \frac{C_{oc}}{C_w} \tag{2.8}$$

K_{oc} is related to the overall partition coefficient K_d by the fraction or organic carbon (OC) present in the soil as in Equation (2.9) [33].

$$K_d = f_{oc} K_{oc} \tag{2.9}$$

Secondly when the fraction of organic carbon (i.e. f_{oc}) is less than ($5x10^{-2}$ kg_{oc}/kg_{solid}), sorption to the surfaces of the clay minerals is often the major sorption mechanism [36]. Nitroaromatic compounds sorb specifically and reversibly to natural clay mineral surfaces depending on their abundance and degree of saturation of weakly hydrated cations such as K^+ and NH_4^+, but strongly hydrated cations such as H^+, Na^+ and Ca^{2+}, Al^{3+} prevents specific interaction [36]. Nitroaromatic compounds having nitro groups or other electron withdrawing substituents that are in resonance with the aromatic ring form coplanar electron donor–acceptor (EDA) complexes (n $\rightarrow \pi$ – complex) with

oxygen ligands present at the external siloxane surface(s) of clay minerals [36]. Only phyllosilicates among the various naturally occurring minerals are capable of forming strong EDA complexes [37]. The affinity and adsorption capacity of the clays for Nitroaromatic compounds (NACs) increase in the order kaolite < illite < montmorillonite [36]. In contrast to the measurement of f_{oc} in solid matrices, a reliable analytical method to determine the amount of siloxane sites that are available for specific adsorption of NACs does not exist [38].

The structure of NACs greatly affects their ability to form electron donor-acceptor complexes. Planar NACs with a highly electron deficient π-electron system due to the presence of several electron-withdrawing and electron delocalizing substituents show the highest adsorption [38]. Orthosubstituents such as alkyl, halogen, or even nitro groups decrease the affinity of adsorption of NACs to the clay minerals by preventing coplanarity [39].

The potential of strong and reversible retention of NACs due to specific adsorption to clay minerals may have some significant implications for remediation measures natural attenuation and the choice of remediation schemes at the contaminated sites. The mobility of NACs may be controlled by manipulating the K^+- saturation of the clay mineral present. On one hand, a ready release of NACs may be desirable as in case of ex-situ treatments of the sediments or *in situ* pump and treat remediation schemes. But on the other hand, immobilization of NACs due to enhanced adsorption may be considered to protect the groundwater [38].

2.2. Nitrification

2.2.1. Background

Nitrification is the biological oxidation of reduced forms of inorganic nitrogen to nitrite or nitrate. Nitrite is converted to nitrate (the most oxidized form) exclusively by prokaryotes. It is usually found in trace amounts in aerobic habitats and only accumulates at low oxygen partial pressures (e.g., in soil with high water potential). Because of the toxicity of nitrite for living organisms, the maintenance of low nitrite concentrations in aerobic habitats is essential. Under aerobic conditions nitrite is converted to nitrate by nitrifying bacteria [40].

Nitrite oxidizers are gram-negative bacteria and are characterized by the prefix *Nitro-*. They are further subdivided into four genera – *Nitrobacter, Nitrococcus, Nitrospira* and *Nitrospina*. Lithotrophic nitrite oxidizers are autotrophs i.e. they fix carbon dioxide (CO_2) via the Calvin-Benson Cycle [41] and to a lesser extent, via phosphoenolpyruvate carboxylase [42]. They are also capable of growing mixotrophically with nitrite as electron donor and with a combination of CO_2 and organic compounds as carbon source. Compared to purely autotrophic growth, the addition of organic compounds stimulates cell growth and increased cell yield [43-46]. Furthermore nitrite oxidizers like *Nitrobacter winogradskyi, N. hamburgensis and N. vulgaris* can grow chemoorganotrophically with acetate or pyruvate as electron donor and dioxygen or nitrate as electron acceptor [47-49]. However for these organisms, heterotrophic growth was always slower than lithotrophic growth. Except *Nitrobacter*, all other isolated nitrite oxidizers are obligate lithotrophs [50].

Nitrifying bacteria are aerobic with optimal activity at mesophillic temperatures and neutral to alkaline pH values (6.5 -8.5). They are slow growing organisms because their cell growth is inefficient. The genus *Nitrobacter* has been the concentration of studies as it is the only genus that has been isolated from soil except one strain of *Nitrospira* that was isolated by Bock *et. al.*, [51].

2.2.2. Stoichiometry of Nitrite oxidization

Nitrite oxidation is carried out by the membrane bound soluble enzyme nitrite oxidoreductase [52] with oxygen supplied by water [53-55] and two electrons released for energy generation as shown in Equation (2.10) and (2.11):

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (2.10)$$

$$0.5O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad (2.11)$$

Combining Equation 2.9 and 2.10 gives the final Equation (2.12)

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (2.12)$$

The produced nitrate is inhibitory for *Nitrobacter* species at concentrations between 30 and 65 mM [56].

CHAPTER 3

BADGER ARMY AMMUNITION PLANT

The information provided herein briefly documents the history and problems of DNT contamination in the Settling Ponds and Spoils Disposal Areas at Badger Army Ammunition Plant, followed by a summary of key concerns based on these prior investigations raised by the Development of Site-Specific Soil Residual Contaminant Levels proposal submitted in May of 2002.

Badger Army Ammunition Plant (BAAP) was constructed in 1942 to produce propellants for use in cannon, rocket and small arms ammunition for World War II. The Settling Ponds and Spoils Disposal Areas are located along the southern boundary of Badger Army Ammunition Plant. There are four Settling Ponds and five Disposal Areas along with Final Creek and the Main Ditch from the rocket production areas. The Settling Ponds (~55 acres) were used for aeration and settling basins for the treated effluent from production wastewater. Sediments in the ponds were removed via dredging between 1973 to the late 1970's, and were placed in the Spoils Disposal Areas (~13 acres). Production of explosives was terminated in 1975, and remedial investigation and activities associated with contaminated soil and groundwater started in 1988. Currently the Settling Ponds Areas are covered with vegetation with only a few acres filled with water in Settling Pond 1 [57].

Recent investigations by Olin have determined the extent of DNT contamination in various locations of the Settling Ponds and Spoils Disposal Areas. Results from these extensive sampling efforts indicate that concentrations of both 2,4-DNT and 2,6-DNT are

(a)

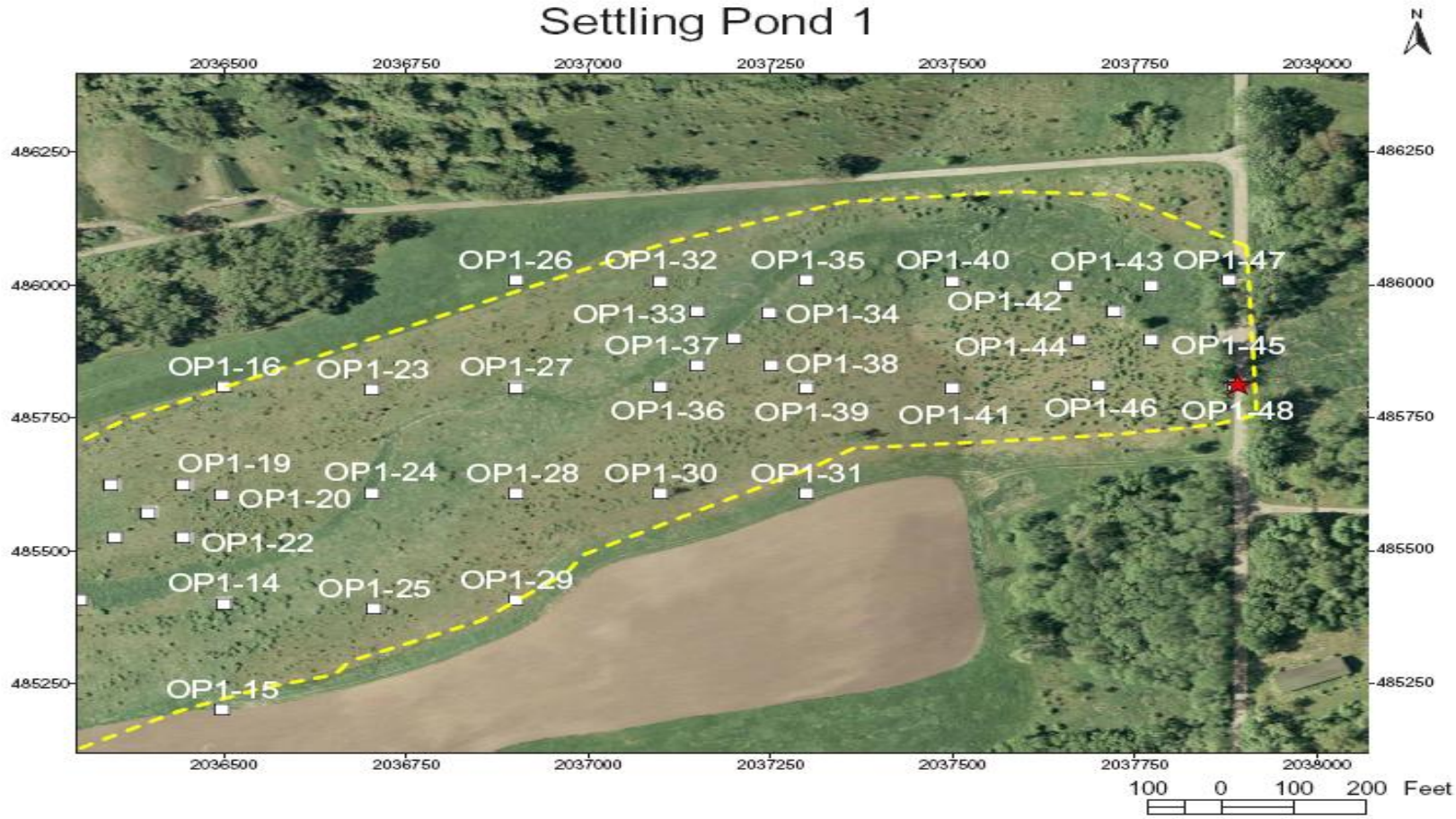


Figure 3.1.Site map of BAAP: (a) Settling Pond 1 (OP1); (b) Settling Pond 4 (OP4); (c) Spoils Disposal Area (SSB). The squares indicate the sampling points. Star in red indicates the position of the sampling point of the soil sample used in this study.

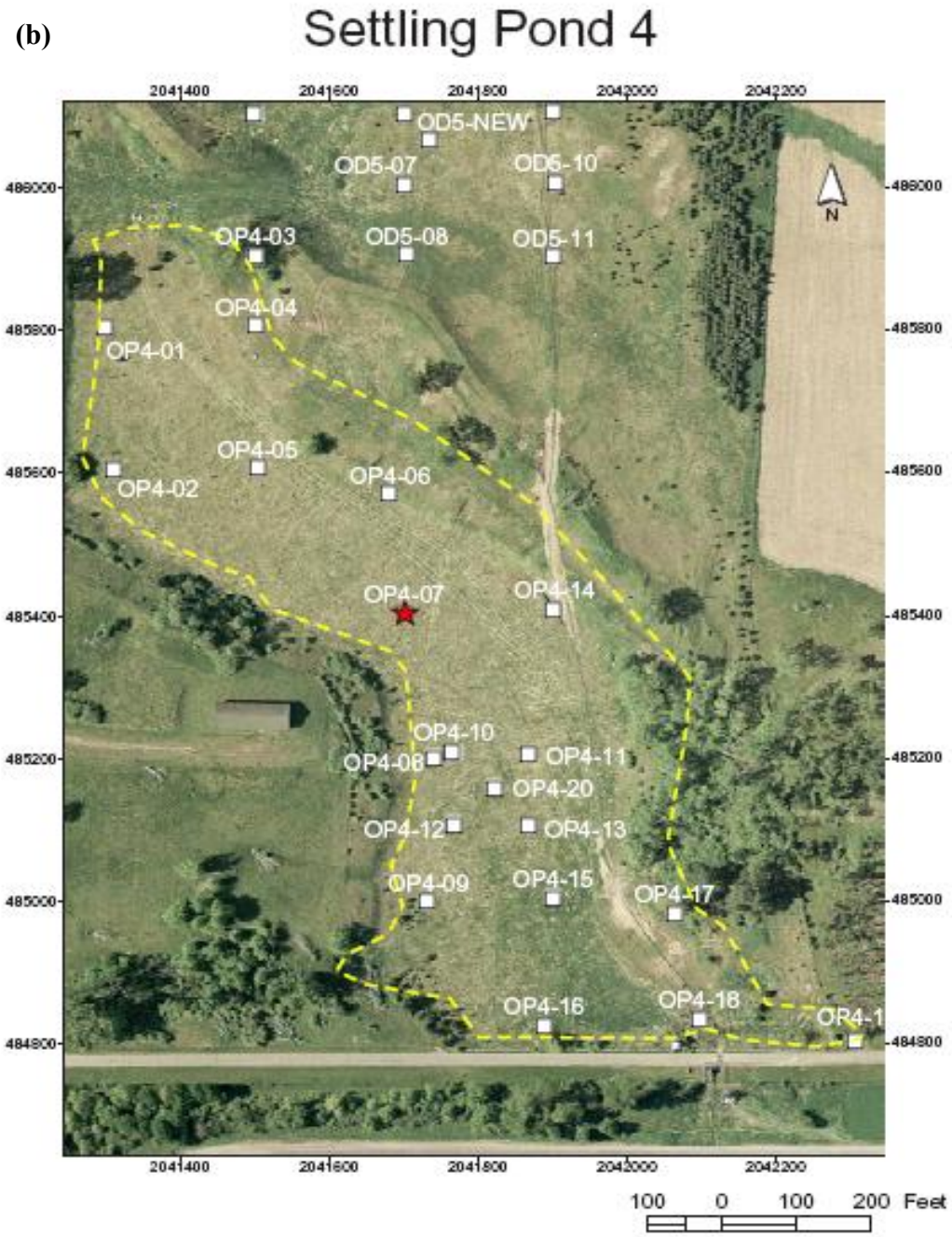


Figure 3.1. Site map of BAAP: (a) Settling Pond 1 (OP1); (b) Settling Pond 4 (OP4); (c) Spoils Disposal Area (SSB). The squares indicate the sampling points. Star in red indicates the position of the sampling point of the soil sample used in this study.

(c)

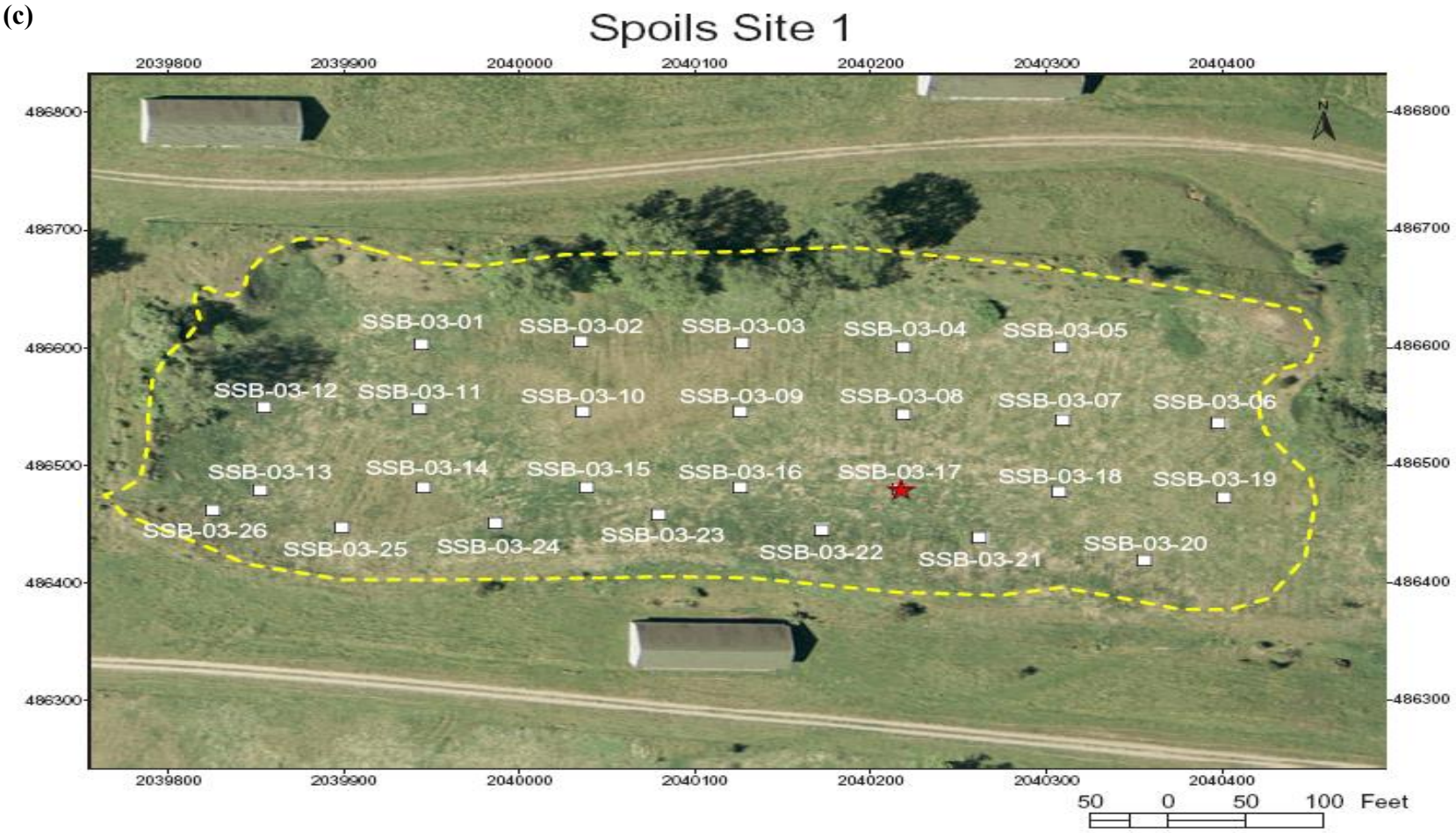


Figure 3.1. Site map of BAAP: (a) Settling Pond 1 (OP1); (b) Settling Pond 4 (OP4); (c) Spoils Disposal Area (SSB). The squares indicate the sampling points. Star in red indicates the position of the sampling point of the soil sample used in this study.

very low – generally several orders of magnitude lower than the contaminated soils from the Propellant Burning Ground (up to 28% by weight). For instance, the concentrations of 2,4-DNT were 1.9–470, 0.64–110, and 660 mg/kg in the Spoils Disposal Areas, Settling Ponds, and Final Creek, respectively. For 2,6-DNT, the maximum concentrations were 0.11– 32, ND–6.80, and 41 mg/kg in the Spoils Disposal Areas, Settling Ponds, and Final Creek, respectively. Several areas have soils contaminated with 2,4-DNT above the direct contact Residual Contact Level (RCL) of 160 mg/kg. DNT concentrations as reported by BAAP at various sampling locations at the Settling Ponds and Spoils disposal area are shown in Table 3.1.

The development of site-specific Soil Residual Contaminant Levels proposal predicted the DNT concentration in underlying groundwater in the Settling Ponds and Spoils Disposal Areas using existing fate and transport models (SESOIL and AT123D). Models predicted that DNT would leach into groundwater at or close to the water solubility concentration. This model prediction, however, is not consistent with site observations. The Wisconsin Department of Natural Resources Administrative Code, Chapter NR 720.19 discourages the use of models for determination of biodegradation rates and pathways in deference to actual field data. The models overestimate the DNT concentration in the groundwater, mostly likely due to the omission of certain fate processes in the model. Specifically, these processes include:

1) aerobic biodegradation of DNT in the surface soils and the vadose zone, and
2) sorption behavior of DNT.

Table 3.1. Historical Site Sampling Data (as reported by BAAP)

A. Settling Pond 1: OP1-48

Depth	2,4-DNT (mg/kg)	2,6-DNT (mg/kg)	DPA (mg/kg)	Soil Characteristics
1.5 Feet	52	6.8	20	yellowish red fine to medium sandy silt
3 Feet	6.8	ND	0.096	yellowish red fine to medium sandy silt

* Sample was collected 12-10-1997.
* Data comes from the report "Field Sampling report: Settling Ponds & Spoils Disposal Areas Badger Army Ammunition Plant" dated May 21, 2001.

B. Settling Pond 4: OP4-07

Depth	2,4-DNT (mg/kg)	2,6-DNT (mg/kg)	DPA (mg/kg)	Soil Characteristics
1.5 Feet	1.6	ND	1.2	very dark gray organic silt with fine sand; small (1/2' diameter) inclusions
3 Feet	ND	ND	ND	very dark gray organic silt with fine sand; small (1/2' diameter) inclusions

* Sample was collected 12-9-1997.
* Data comes from the report "Field Sampling report: Settling Ponds & Spoils Disposal Areas Badger Army Ammunition Plant" dated May 21, 2001.

C. Spoil Site 1: SSB-03-17

Depth	2,4-DNT (mg/kg)	2,6-DNT (mg/kg)	2-NDP (mg/kg)	DPB (mg/kg)	DPA (mg/kg)	NG (mg/kg)	Soil Characteristics
1 Foot	0.22	ND	ND	3.25	0.96	5.89	black organic silt
3 Feet	0.57	ND	0.09	9.68	1.06	ND	dark brown clay with iron streaks in seams
5 Feet	13.0	2.49	1.98	77.1	17.3	3.94	dark brown clay with iron streaks in seams
7 Feet	ND	ND	ND	ND	ND	ND	brown coarse sand with gravel
9 Feet	ND	ND	ND	0.39	0.47	0.351	brown coarse sand with gravel
11 Feet	ND	ND	ND	ND	ND	1.74	brown coarse sand with gravel

* Sample was collected 1-28-03.
* Field observations suggest that the spoils are approximately 5.6 feet thick at this location.
* Data comes from laboratory reports and field notes.

CHAPTER 4

MATERIALS AND METHODS

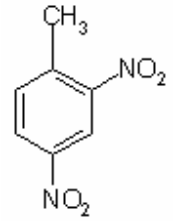
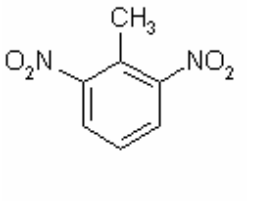
4.1. Chemicals

2,4-DNT (97%) and 2,6-DNT (98%) (Table 4.1) were obtained from Sigma-Aldrich. The following reagent grade chemicals were used for mineral media constituents: $CaCl_2.2H_20$, $CaSO_4.5H_2O$, $FeSO_4.7H_2O$, H_3BO_3, K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, NaCl, $Na_2MO{\cdot}6H_2O$, and $ZnSO_4{\cdot}7H_2O$ (reagent grade, Fisher Scientific, Acros) [22]. Acetonitrile (HPLC grade, 99.9%, Fisher Scientific), reagent grade trifluoroacetic anhydride (Fisher Scientific) and acetic acid (Acros, glacial reagent) were used for HPLC analysis. A solution containing 1.8 mM $NaHCO_3$ plus 1.7 mM of Na_2CO_3 (Dionex) was used for IC analysis. $NaNO_2$ and KNO_3 (Fisher Scientific) were used for preparing calibration curve for NO_2^- and NO_3^- analysis respectively. NaOH (1N) was used to adjust pH (8 – 8.5) of the mineral media as required.

4.2. Soil Samples

Soil cores analyzed in this study were collected at BAAP from Settling Pond 1 at depth 0-3 feet, labeled **OP1**; Settling Pond 4 at a depth of 0-3 feet, labeled **OP4** and from Spoils Site 1 at depth of 3-6 feet, labeled **SSB** and were shipped to us. Upon arrival the soils were homogenized and debris removed by hand (pebbles, plant matter, etc.). The soil samples were transferred into stainless steel containers and stored at 4°C. The soils were air-dried, ground, and sieved (2 mm) for microcosm, column and sorption studies. Soil samples were analyzed for its characteristics by the Department of Crop and Soil Science, University of Georgia, at their commercial laboratory for environmental

Table 4.1. Chemical Properties of 2,4-DNT and 2,6-DNT

Properties	**2,4-DNT**	**2,6-DNT**
Chemical Formula	$C_7H_6N_2O_4$	$C_7H_6N_2O_4$
Chemical Structure		
Molecular Weight (g/mole)	182.1	182.1
Aqueous Solubility (mg/L)	280 (25°C) [a] 270 (22°C) [b]	208 (25°C) [a]
Log K_{ow}	1.98 [a, b]	2.02 [a] 1.89 [a, b]
Log K_{oc}	2.40 [a, b] 1.94 [c]	1.89 [a, b] 2.00 [c]

[a] [58],[b] [59], [c] [60]

analysis. The results of soil properties and methods of analysis are summarized in Table 4.2.

4.3. Sample Analysis

All aqueous samples taken in the study were filtered through a PTFE filter unit (0.22 µm, Millipore) prior to analysis. Separation and quantification of DNT was by HPLC (Agilent) equipped with a diode array detector. Two HPLC methods were used throughout the project. DNT was separated by either using a Zorbax® SB-C18 with a mobile phase of 68:32 (water (with 0.1% acetic acid):acetonitrile) at a flow rate of 1 mL/min or a Hybercarb® porous graphite column (100 x 3 mm, 5µm, Thermo Hypersil, UK) with mobile phase of 96:4 (acetonitrile : water (0.55% trifluoroacetic anhydride)) at a flow rate of 0.7 mL/min [21]. For both methods, 2,4-DNT was quantified at 246 nm and 2,6-DNT at 230 nm. For each batch of samples analyzed a new calibration curve was performed using known 2,4-DNT and 2,6-DNT standards to assure the data quality and consistency. Method detection limit for DNT was approximately 0.02 mg/L and was obtained by following Standard Methods for Wastewater Treatment method 1030.E. [61]. Nitrite was measured colorimetrically and using ion chromatography (Dionex IC) following Standard Methods for Wastewater Treatment method 4500-NO2 [61]. Nitrate was measured using ion chromatography (Dionex IC) following Standard Method for Wastewater Treatment method 4500-NO3 [61]. Nitrite and nitrate were extracted from the soil samples following Method of Soil Analysis method 33.3 [62]. pH was measured with a Denver Instrument pH 220.

4.4. DNT Soil Extraction Procedure

For each homogenized soil (OP1, OP4 and SSB), five sub samples (approximately 0.05 g

Table 4.2. Soil Physical and Chemical Properties

Physical parameters	OP1	OP4	SSB
pH[a]	5.7	6.2	6.3
Moisture (wt/wt %)	1.35	3.45	5.25
EC (meq/100 g)[b]			
Na	0.02	0.02	0.04
Mg	1.12	1.71	2.65
K	0.12	0.19	0.23
Ca	3.6	14.99	36.35
CEC (meq/100 g)[c]	7.28	19.46	26.09
Texture Distribution, %[d]			
Clay	8.36	17.16	13.52
Silt	10.43	74.64	49.96
Sand	81.21	8.2	36.52
Soil Type (USDA)[e]	Loamy Sand	Silt Loam	Loam
Elemental Analysis (ppm)			
TOC (wt/wt %)[f]	0.179	1.982	3.346
Ammonia N[g]	5	14	15
Nitrate N[h]	24.68	2.26	12.44
Nitrite N[i]	0.12	0.49	1.11
Phosphate[j]	140	191	197
Mn[k]	502	286.3	696.8
Fe[k]	9980.7	13405.9	6621.5
Cu[k]	21.3	15.7	59.7
Zn[k]	119.9	65.8	86.3
Mo[k]	0.18	0.19	0.62

Methods used for the analysis of soil samples:

[a] pH using combined calomel glass electrode. [b]EC was determined using ammonium acetate method, Methods of Soil Analysis Part II- Chemical and Microbiological Properties, Second edition, Agronomy #9, ASA. Page 160. [c] CEC was done by displacing the cations on the soil with 1 M K acetate solution. The K is then displaced with 1 M ammonium acetate. K ions were determined by ICP/MS. [d] Texture was determined using hydrometer procedure, Black, C.A. (eds.), Methods of Soil Analysis. Part 1. Am. Soc. Agron., Madison, WI, 1965. [e] Based on USDA standard classification [f] TOC was determined by ignition in a LECO CNS 2000 analyzer (LECO Corporation, St. Joseph, MI 49085-2396. Prior to ignition, the samples were treated with 10% HCl to decompose inorganic carbon and dried at 105°C. [g] Ammonia N colorimetrically. [h] Nitrate was done by ion chromatography. [i] Nitrite N was done by flow injection analysis using OI Analytical analyzer. [j] Phosphate was done colorimetrically with the molybdate blue reaction. [k] Elements analysis: Soils were digested using EPA Method 3051: "Microwave assisted acid digestion of sediments, sludges, soils, and oils" The digestate was analyzed for the required elements using EPA Method 200.8". Determination of trace elements in waters and wastes by inductively coupled plasma - mass spectrometry".

each) were extracted to determine the soil associated DNT concentrations. Using an Ultrafree-MC 0.22 μm two chamber centrifugal filter device (Millipore), soil (0.05 g) and acetonitrile (ACN) (200 μL) was added to the top filter unit and allowed to equilibrate (10 minutes). The unit was centrifuged (5 minutes at 7000 RPM) allowing for separation of the ACN through the top filter unit into the bottom collection unit. This process was repeated twice more for a total of three ACN extractions per soil sub sample [15, 21]. The ACN (600 μL) in the bottom of the collection unit was then analyzed for DNT using HPLC.

4.5. Screening DNT Degrading Activity

All soils were screened for the presence of indigenous microbial populations capable of mineralizing DNT. Shake flasks containing soil sample (5 g) suspended in mineral medium (50 mL) [22] and spiked with either 2,4-DNT (100 mg/L) or 2,6-DNT (100 mg/L) were employed to enrich degraders over a two week period. Abiotic controls were maintained in parallel with sodium azide (1 g/L, as an anti-microbial agent). To assess the presence of specific degraders, samples (1 mL) from both active and control shake flasks were transferred to soil free microcosms containing fresh media and DNT (10 mg/L). Samples from these shake flasks were filtered (0.22 μm, PTFE) and analyzed for DNT, nitrite and pH.

4.6. Batch Sorption Studies

Batch sorption experiments were carried out in Teflon™ centrifuge tubes (PTFE, 50 ml) with soil (3 g), and deionized water (30 ml, pH 7), at various DNT concentration (2.5, 5, 10, 25 and 50 mg/L) based on the method of Haderlein *et. al.* [63]. The samples

were prepared in triplicate for each initial condition. Samples were continuously mixed end over end at approximately 1 rpm at room temperature (22°C). Sodium azide (1 g/L) was added to all the samples to inhibit biological activity. For each soil, controls were prepared with no soil and an initial aqueous phase 2,4- or 2,6-DNT concentration (50 mg/L) to assess other losses from the aqueous phase. Soil free controls demonstrated that other reactions and or losses within the bottles were insignificant (less than 0.1 %). After 24 hrs, to ensure equilibrium [64, 65], each sample tube was centrifuged (1,500 g, 22°C for 20 minutes) and the supernatant was collected and analyzed for aqueous phase DNT concentration using HPLC (as described in section 4.3). Sorbed phase concentrations were calculated by the difference between the initial and equilibrium concentrations as given in Equation (4.1).

$$C_s = \frac{(C_o - C_e)V}{M} \tag{4.1}$$

where, C_s is the mass of sorbed contaminant per mass of soil (mg/kg); C_o and C_e are the initial and equilibrium liquid phase concentration (mg/L), respectively; V is the volume of the liquid phase (L); and M is the mass of soil (kg).

4.7. Batch Desorption Studies

Experimental analysis of soil associated DNT desorption was based on a method modified from Fu *et. al.* [66]. Due to very low or non detectable soil associated DNT in the soil samples provided, an initial adsorption step was necessary for each soil (OP1, OP4 and SSB). Thus adsorption studies carried out with DNT (50 mg/L) were followed by desorption studies. After the solid and aqueous separation (via centrifugation 1,500 g, 22°C for 20 minutes) at the conclusion of adsorption studies, the desorption process was

initiated by replacing 85% of the supernatant with deionized water containing sodium azide (1 g/L). The same solids to volume ratio (1:10) as described above in the sorption protocol (section 4.5) was used. Done in triplicate, the samples were continuously mixed end over end at approximately 1 rpm at room temperature (22°C) for 24 hours. Subsequently, the aqueous and the solid phase were separated (via centrifugation 1,500 g, 22°C for 20 minutes) and the aqueous phase was analyzed for DNT that desorbed from the solid. This process is referred as one desorption step.

As soil SSB was the only soil from the site with detectable levels of associated DNT (30 mg 2,4-DNT/kg soil, determined through solvent extraction procedure as described in section 4.4), it was of interest to determine the desorption rates of the "aged" DNT present in this sample. Desorption was tested using the same method used in spiked soils in centrifuge tubes (PTFE, 50 mL) in duplicate, SSB soil (15 g) and DNT free deionized water (10 mL) containing sodium azide (1g/L) were mixed end over end at approximately 1 rpm at room temperature (22°C) for 5 days (140 hours). The equilibrated system was then centrifuged (5000 rpm, 4°C for 5 minutes), the supernatant was filtered (0.22 μm PTFE) and analyzed using HPLC for desorbed aqueous DNT concentrations.

4.8. Soil Column Studies

Two glass columns (5 cm internal diameter x 20 cm length) with Teflon® endplates (Spectrum Chromatography, Houston, TX) were packed with soil OP1. One column was fed water containing 2,4-DNT alone, and other with 2,6-DNT only. The columns were packed with 2 cm of glass beads (diameter = 3 mm) to prevent wash out of sand and clogging of the effluent line, followed by 10 cm of OP1 soil sample (approximately 300 g) and 5 cm of glass beads (diameter = 6 mm) to uniformly distribute

the influent (Figure 4.1). Simulated rain water (equilibrated to the atmosphere) (10 ml) with a DNT concentration (10 mg/L) was poured into each column once every day to simulate a low concentration influent of DNT into a vadose zone. Both OP1 soil columns were operated for over 3 months; 123 days (116 days of effluent collection) for the 2,4-DNT feed and 116 days (106 days of effluent collection) for the 2,6-DNT feed. Column effluent was collected daily and analyzed for DNT, nitrite and nitrate.

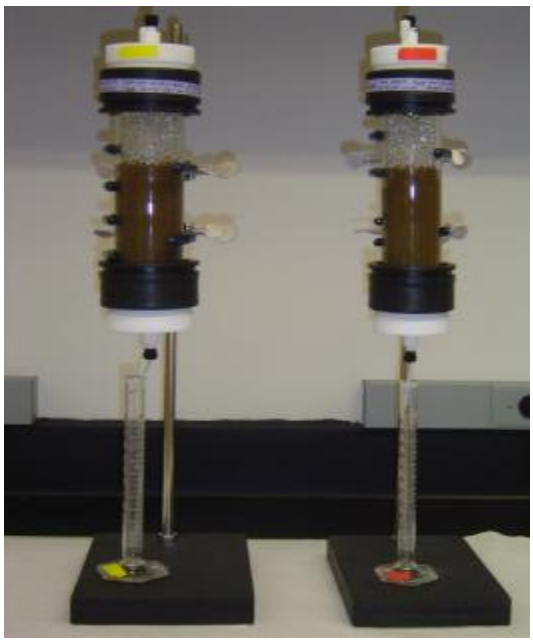
At the conclusion of the study, the columns were dissected and soil bed (10 cm) was divided into four sections starting from the top: (0–2.5 cm), (2.5-5 cm), (5–7.5 cm) and (7.5-10 cm). All four sections were analyzed for residual DNT concentration using the soil extraction procedure (as described in section 4.4). Nitrite and nitrate were extracted from all four sections and measured (as described in section 4.3).

4.9. Screening Activity of Nitrifying Bacteria

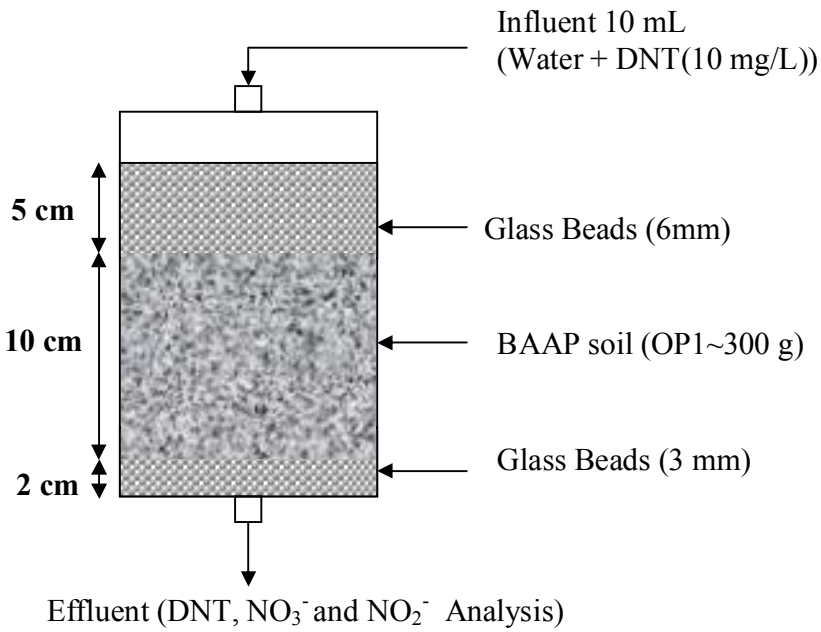
Soil sample OP1 was screened for the ability of indigenous microbial populations to oxidize nitrite that would be evolved from DNT mineralization. Shake flasks containing soil (5 g) taken from each of the four parts of OP1 soil column was suspended in mineral medium (50 ml) [22] spiked with nitrite (100 μM). Samples from the shake flasks were filtered (0.22 μm, PTFE) and analyzed for nitrite disappearance. To assess the production of nitrate, samples (1 mL) from these shake flasks was transferred to soil free microcosms containing fresh media and nitrite (100 μM) after 7 days from the first part of the column (0-2.5 cm).

4.10. Chemostat Study

A continuous growth culture degrading 2,4-DNT was maintained at different



(a)



(b)

Figure 4.1. (a) Soil column reactor: 2,6-DNT (left) and 2,4-DNT (right) (b) Soil column schematic.

temperatures: 22°C, 15°C, 10°C, 7.5°C, and 4°C to study the effect of temperature on the degradation of 2,4-DNT. The bacterial culture was obtained from the batch culture set up from OP1 soil sample that was being maintained in the laboratory (as described in section 4.7). A water bath (Boekel Grant, ORS 200, ± 0.2°C precision) coupled with immersion cooler (Boekel Grant, CS 200) were used to control the desired temperature in the system. The mineral medium [22] spiked with 2,4-DNT (250 μM was fed as the influent with a syringe pump at a flow rate (Q) of 346 mL/day. The volume (V) of the reactor was kept at 860 mL and the hydraulic retention time (θ) was 2.5 days. Influent and effluent were analyzed daily for DNT and nitrite. The temperature was lowered after the steady state at a particular temperature was maintained for three times the retention time.

$$q = \frac{V}{Q} \tag{3.1}$$

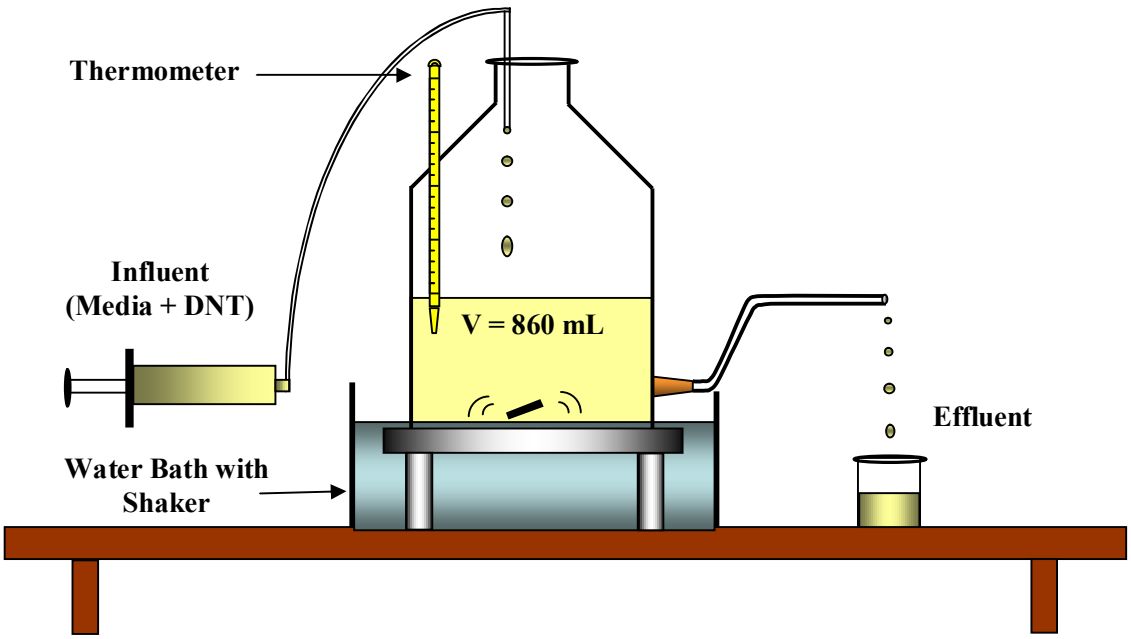


Figure 4.2. Schematic of the chemostat

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Background DNT in Soil

Neither 2,4-DNT nor 2,6-DNT was detected in soil samples from OP1 and OP4. Soil sampled from SSB was found to contain approximately 30 mg/kg (165 μmol/kg) of 2,4-DNT while 2,6-DNT was not detected. Because of the low concentration or even absence of DNT isomers in the soils received, in many experiments the appropriate DNT isomer(s) was added to the soil at known concentrations.

5.2. Microbial DNT Degradation Screening

For all BAAP soils (OP1, OP4 and SSB) indigenous microbes were able to metabolize 2,4-DNT as demonstrated by loss of 2,4-DNT and the corresponding increase in nitrite [8, 13, 22]. For all soils, this activity was transferable. Figure 5.2 shows 2,4-DNT degrading activity of indigenous BAAP cultures after being transferred to fresh media containing 2,4-DNT (70 μM). In contrast, we did not find any BAAP soils (OP1, OP4 and SSB) to be a source of indigenous microbes able to metabolizing 2,6-DNT which is not a contaminant of concern at the site (data not shown).

With the confirmation of the presence of 2,4-DNT mineralizing bacteria the first objective of this work was achieved. The site microbiology appears conducive to the application of monitored natural attenuation (MNA) as a remediation strategy.

5.3. Batch Sorption Studies

All experimentally derived DNT sorption isotherms with BAAP soils were fitted

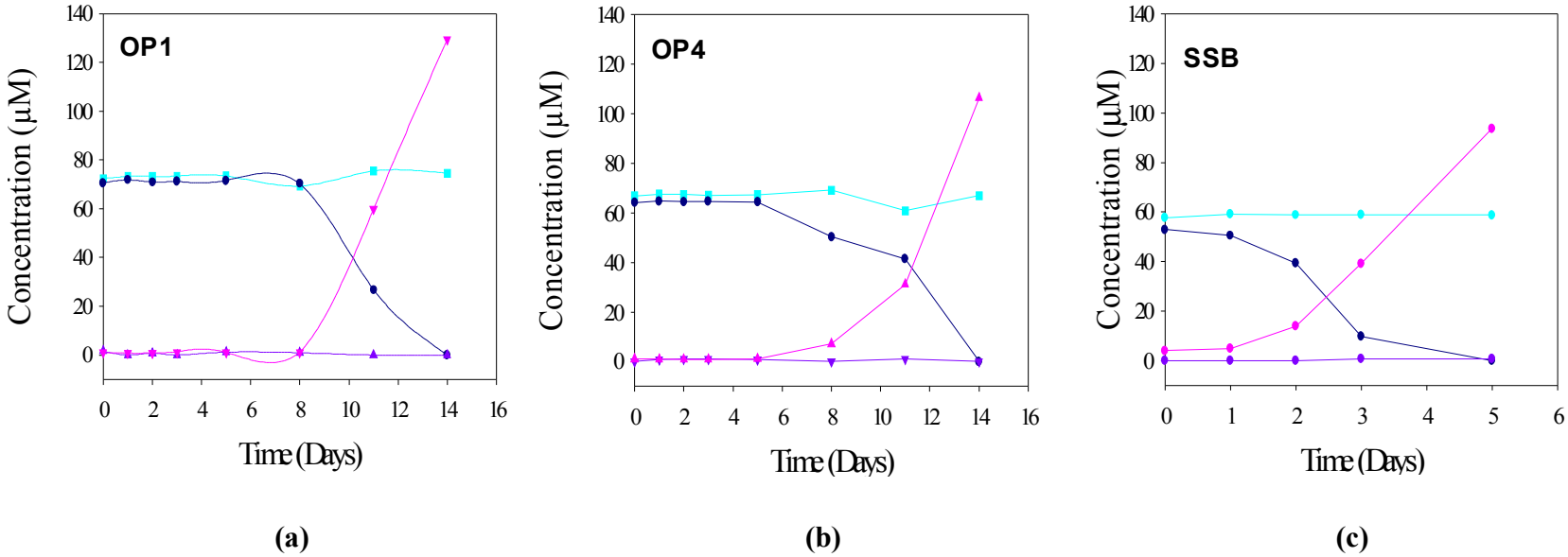


Figure 5.1. Microcosms showing presence of 2,4-DNT Degrading Bacteria in the BAAP Soil Sample (a) OP1-48 (b) OP4-07 (c) SSB 03-17 (■) DNT in control Reactor (●) DNT in Active Reactor (▲) NO_2^- in control Reactor (▼) NO_2^- in Active Reactor.

using Freundlich isotherms by a non-linear regression , where $C_s = K_F \cdot C_w^n$, K_F is the Freundlich capacity coefficient $(mg/kg)/(mg/L)^n$, Freundlich coefficient, $n < 1$, except 2,6-DNT adsorption with soil OP1 which was fitted using a linear isotherm, $C_s = K_d \cdot C_w$, K_d is the linear distribution coefficient (L/kg) (Figure 5.1). 2,4- and 2,6-DNT behaved similarly for each soil except for soil OP1 (Figure 5.2, Table 5.1). Derived K_F values varied in magnitude depending upon the soil properties (Table 4.2). For example soil OP1 with high sand (80 %), low clay, low OC content and, low cation exchange capacity had relatively low K_F values (K_F = 6.55 L/kg, n = 0.74 (Freundlich) for 2,4-DNT; K_d = 1.21 L/kg (linear) for 2,6-DNT) when compared to soil SSB with low sand, high clay, moderate amount of OC content and, high cation exchange capacity (K_F = 342.45 L/kg, n = 0.59 for 2,4- DNT; K_F = 278.374 L/kg, n = 0.57 2,6-DNT). Soil OP4 which has clay content comparable to that of SSB and OC content between OP1 and SSB displayed DNT isotherms and K_F values (66.6 L/kg for 2,4-DNT and 32.9 L/kg for 2,6-DNT) between those observed for OP1 and SSB.

The observation of strong association of DNT with soil SSB having low sand, high clay and high cation exchange capacity and weak association of DNT with soil OP1 having high sand, low clay and low cation exchange capacity were supported by the soil characteristics (Table 4.2) [36-39].

5.4. Batch Desorption Studies

All experimentally derived DNT desorption isotherms with BAAP soils were fitted using Freundlich isotherms by a non-linear regression (as described for adsorption isotherm in section 5.3). 2,4- and 2,6-DNT showed similar desorption with each soil (Figure 5.1). For example, with BAAP soil SSB over the course of 13 desorption steps,

2,4- and 2,6-DNT desorbed quite similarly. Similar to adsorption, desorption of DNT from BAAP soils depended on individual soils. Soil SSB which sorbed much more DNT than soil OP1 and OP4, demonstrated lower extent of desorption per step and continued to desorb DNT for more steps than OP1 and OP4. Soil SSB, DNT concentrations at the beginning of the desorption study were 2,770 μmol/kg of 2,4-DNT and 2,350 μmol/kg of 2,6-DNT which after 13 desorption steps were reduced to 1960 μmol/kg of 2,4-DNT and 1260 μmol/kg of 2,6-DNT was. Soil OP4 desorbed all detectable DNT after 9 steps and showed desorption along the same isotherm as adsorption with 92 % of 2,4-DNT and 98 % of 2,6-DNT was recovered through desorption in the aqueous phase. Soil OP1 the desorption of 2,6-DNT was fitted by Freundlich isotherm unlike adsorption which was fitted by linear isotherm. Desorption of 2,6-DNT (K_F = 223.7 L/kg) was less extensive than 2,4-DNT (K_F = 41.1 L/kg) (Table 5.1).

For reasons that probably control the K_F discussed in Chapter two, desorption appears to be a factor of soil type and properties of DNTs. Nitroaromatic compounds tend to sorb specifically and reversibly to natural clay mineral surfaces depending on their abundance and degree of saturation of weakly hydrated cations such as K^+ and NH_4^+ [36-39]. SSB with higher clay, cation exchange capacity and OC content thus demonstrates a desorption process that slowly desorbs DNT concentration in the aqueous phase over many steps (which can be thought of as pore volumes in a soil).

Soil SSB was the only soil received with a detectable level of 2,4-DNT (approximately 30 mg/kg) (as described in section 5.1). However, when SSB was analyzed for desorption of this DNT; no DNT was observed to desorb into the aqueous phase after 5 days, indicating a strong association with the soil. This might be

Table 5.1. K_F values (Freundlich model) determined experimentally for 2,4-DNT and 2,6-DNT for BAAP soils

Soil Sample	2,4-DNT				2,6-DNT			
	Adsorption		Desorption		Adsorption		Desorption	
	K_F (L/kg)	n	K_F (L/kg)	n	K_F (L/kg)	n	K_F (L/kg)	n
OP1 (0-3 ft)	6.6 ± 2.5	0.74	41.1 ± 3.8	0.41	NA*	1	223.73 ± 1.4	0.4
OP4 (0-3 ft)	66.6 ± 4.5	0.54	83.4 ± 10.6	0.50	32.9 ± 2.7	0.55	37.3 ± 2.4	0.53
SSB (3-6 ft)	342.5 ± 22.0	0.59	1844.5 ± 34.5	0.11	278.4 ± 23.1	0.57	986.6 ± 19.0	0.23

Test conditions: soil: water = 1:10 (w/v), temperature 22°C, 1g/L NaN_3
NA = Not Applicable
*2,6-DNT showed linear adsorption with OP1 soil, K_d = 1.21 L/kg

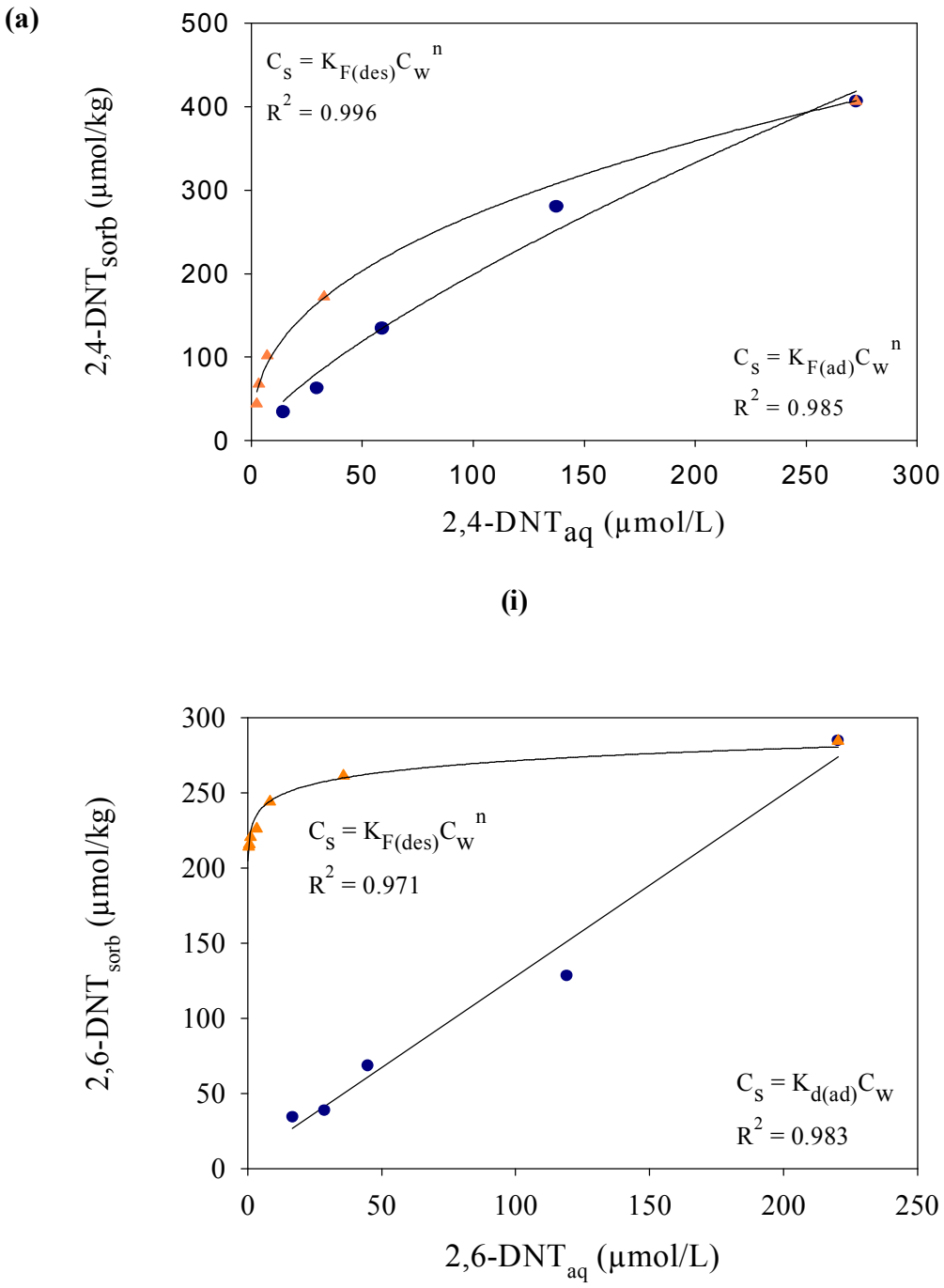
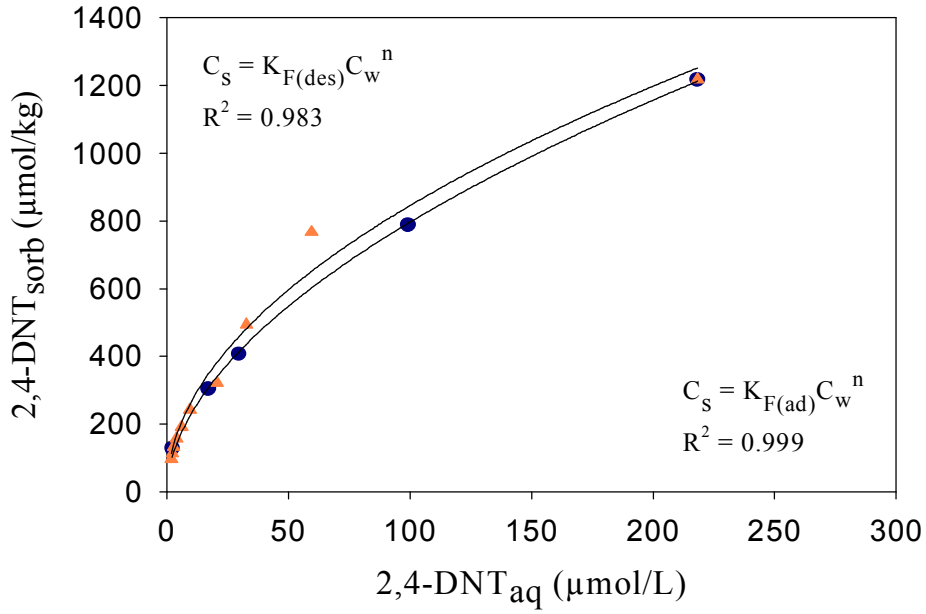
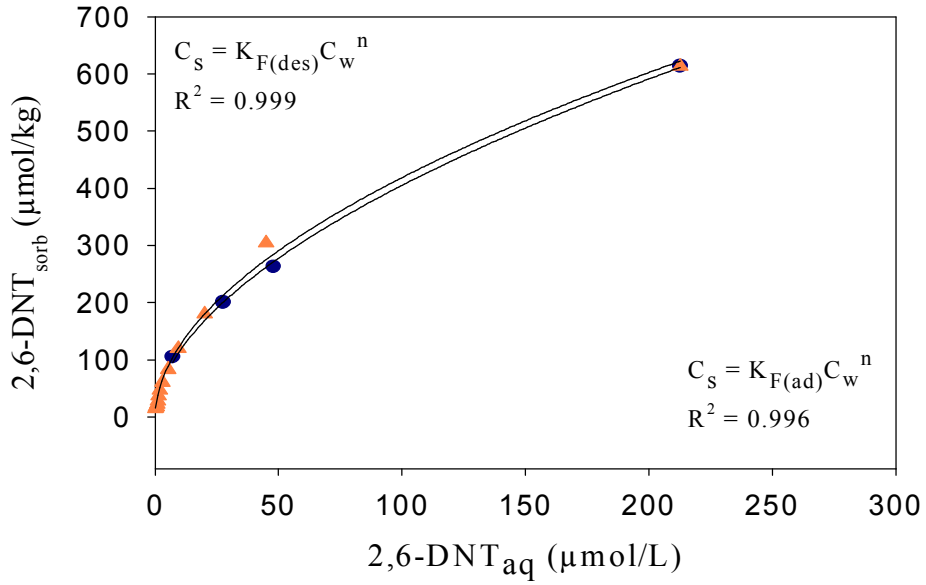


Figure 5.2. Sorption and desorption isotherms of BAAP soils (a) OP1 (b) OP4 (c) SSB; (i) 2,4-DNT and (ii) 2,6-DNT. (●) represents adsorption data points for 2.5, 5, 10, 25, and 50 mg/L as initial aqueous DNT concentration; (▲) represents desorption data on the soil sample with highest concentration of adsorbed DNT

(b)



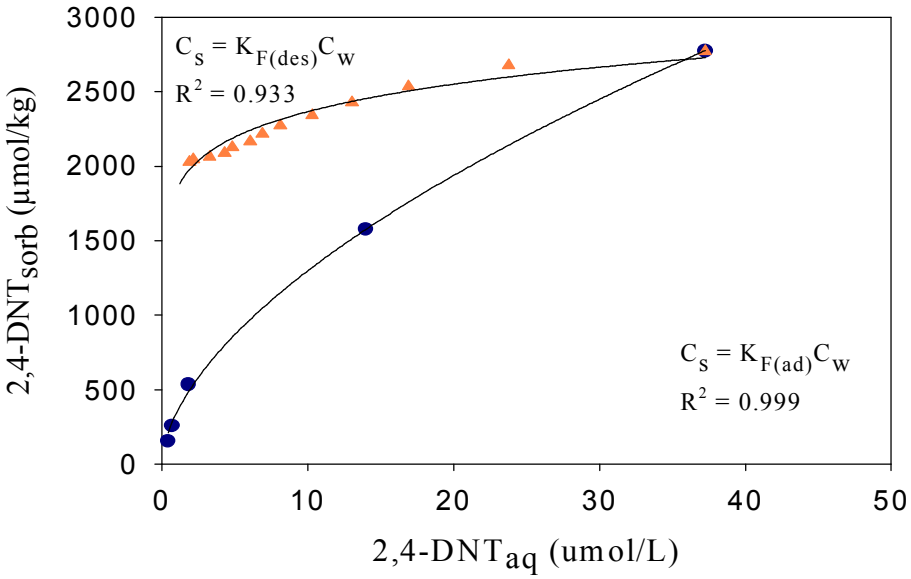
(i)



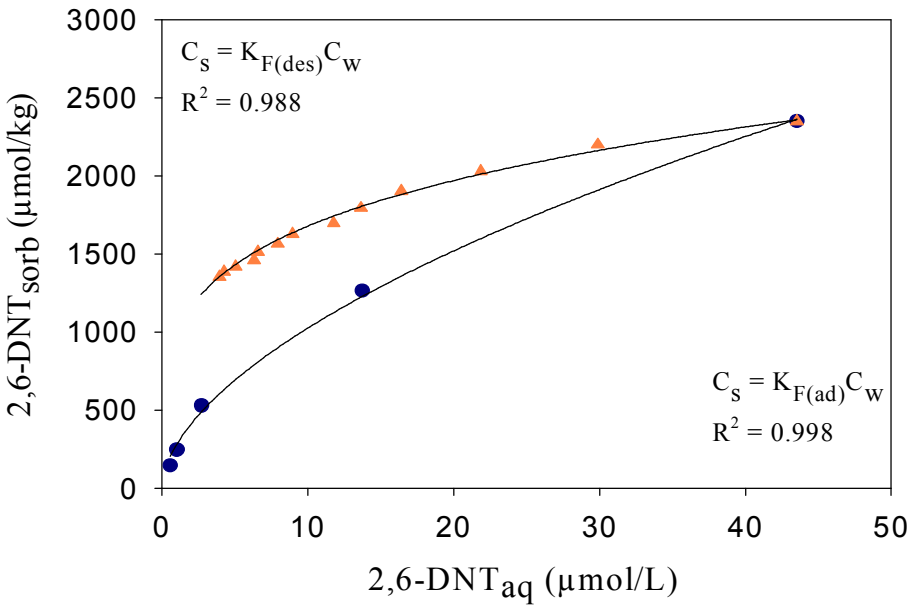
(ii)

Figure 5.2. Sorption and desorption isotherms of BAAP soils (a) OP1 (b) OP4 (c) SSB; (i) 2,4-DNT and (ii) 2,6-DNT. (●) represents adsorption data points for 2.5, 5, 10, 25, and 50 mg/L as initial aqueous DNT concentration; (▲) represents desorption data on the soil sample with highest concentration of adsorbed DNT.

(c)



(i)



(ii)

Figure 5.2. Sorption and desorption isotherms of BAAP soils (a) OP1 (b) OP4 (c) SSB; (i) 2,4-DNT and (ii) 2,6-DNT. (●) represents adsorption data points for 2.5, 5, 10, 25, and 50 mg/L as initial aqueous DNT concentration; (▲) represents desorption data on the soil sample with highest concentration of adsorbed DNT.

due to longer equilibrium time experienced by the aging process of contaminated soils [67-69].

5.5. Soil Column Studies

5.5.1. 2,4-DNT Column

With an influent of simulated rainwater (10 mL) fed every day at an average concentration of 9.4 ± 2.3 mg/L of 2,4- DNT, the total volume of column influent throughout this study was 1.2 liters of solution with a total mass of 2,4-DNT applied of 11.1 mg (60.9 μmoles). Over the course of the study (123 days), 2,4-DNT was never observed in the effluent. An initial peak of nitrite (Figure 5.3(a)) was, presumably due to the wash out of nitrite already present in the soil or the nitrite oxidizers being in the lag phase and not utilizing the nitrite evolved upon 2,4-DNT degradation, or both. After the initial washout, nitrite was detected in the effluent but at levels lower than expected stoichiometric release (3-5 μM instead of 100 μM) calculated from the expected biodegradation of all 2,4-DNT applied. Trace levels of nitrate were detected occasionally in the effluent (3-5 μM).

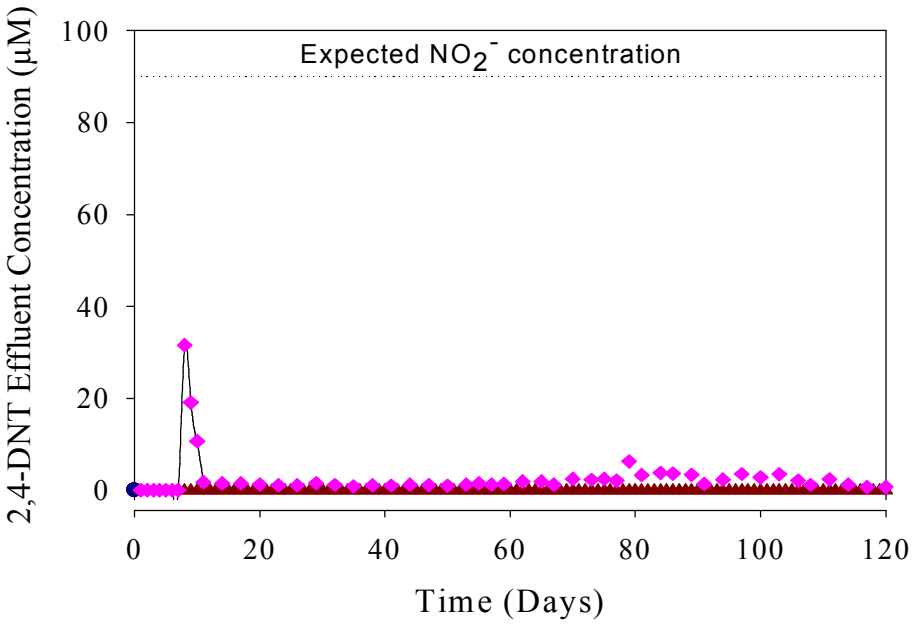
At the completion of the study the column was dissected and fractions were subjected to soil extraction procedure (as described in section 4.3). No sorbed 2,4-DNT was detected in any sample. Trace levels of nitrite and nitrate (3-5 μM) were detected. Based on these observations, it was evident that 2,4-DNT was being biodegraded in the column. To assess the kinetics of degradation, a simple, steady state first order equation describing apparent 2,4-DNT loss was used to obtain an estimate of the rate coefficient using Equation (5.1).

$$S = S_o e^{-kt} \tag{5.1}$$

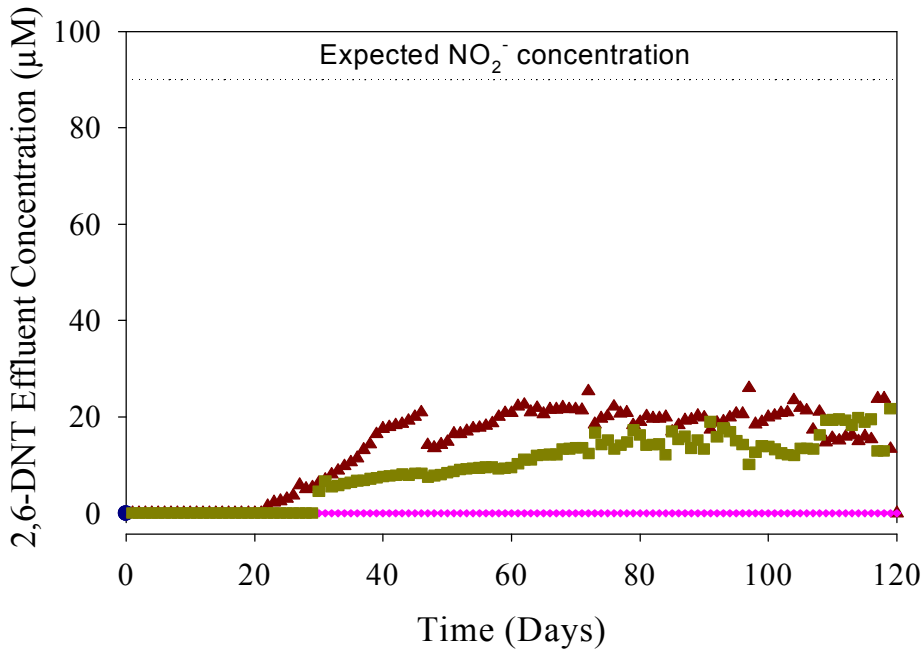
where, S_o is the initial substrate concentration (9.4 mg/L), S is the effluent substrate concentration at steady state and t is the hydraulic retention time (8 days, based on the breakthrough of water from the soil column). Because the effluent concentration was below the detection limit (0.2 mg/L), it was assumed to be one-half the detection limit (0.1 mg/L). The first order rate constant obtained from this analysis was $\mathbf{k \geq 0.57\ d^{-1}}$. No 2,4-DNT was found to be associated with the soil OP1 upon dissection and thus sorption on the soil was ignored while calculating the first order rate constant.

5.5.2. 2,6-DNT Column

With an influent of simulated rainwater (10 mL) fed everyday at an average concentration of 9.4 ± 2.1 mg/L of 2,6-DNT, the total volume of column influent in the study was 1.06 liters of solution with a total applied mass of 2,6-DNT was 10.7 mg (58.5 μmoles). On day 22, 2,6-DNT was detected in the effluent and reached steady state value near day 41. From day 41 to day 106 the average 2,6-DNT concentration was approximately 3.6 ± 0.4 mg/L. But over the course of the study, 2,6-DNT effluent concentration never reached the influent concentration. At low levels, an unknown compound was consistently detected in the effluent. UV/Vis spectral analysis suggested it to be a reduced form of 2,6-DNT (an amine). A number of amines were analyzed on the HPLC and based on retention time and UV/Vis spectra the identity of the compound was confirmed to be 2-methyl-3-nitroaniline, a reduced product of 2,6-DNT. As discussed in Chapter 2, such non-specific reduction has been observed in previous studies [8].

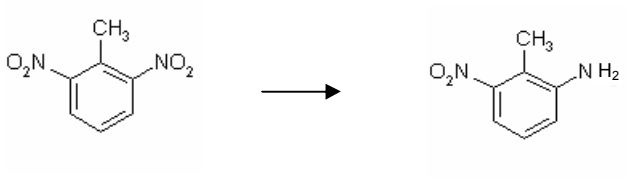


(a)



(b)

Figure 5.3. (a) 2,4-DNT soil OP1 column effluent concentration vs. time (b) 2,6-DNT oil OP1 column effluent concentration vs. time ; (▲) observed DNT concentrations (♦) observed nitrite concentrations; (■) observed 2-methyl-3-nitroaniline concentrations.



2,6-DNT

2-methyl-3-nitroaniline

In contrast to the 2,4-DNT column an initial nitrite peak due to the wash out of the nitrite was not detected in the 2,6-DNT column (Figure 5.3 (b)) because samples were not collected for the first few days of the study. The fate and transport of the reduced compound was not evaluated. Over the course of the study, neither nitrite nor nitrate was detected in any effluent samples.

At the completion of the study the column was dissected and the column soil was subjected to the soil extraction procedure (as described in section 4.3). 11.4 ± 0.2 mg/kg of 2,6-DNT was detected to be adsorbed to these soils. No nitrite and nitrate were detected and 55.4 % of the 2,6-DNT added as influent was recovered in the effluent (32.9 % as 2,6-DNT and 22.5 % as 2-methyl-3-nitroaniline). The lack of mass balance closure might be because of the sorption, reactions, or both, undergone by the transformation products or other transformation products being evolved but not detected by the method used for HPLC analysis.

5.6. Screening Activity of Nitrifying Bacteria

Nitrite is evolved during degradation of DNT and its production has been used as an indicator of DNT degradation [8, 12, 16, 22, 70]. While complete 2,4-DNT degradation was maintained throughout the study (123 days), stoichiometric production of nitrite, was not observed in the effluent from the column. (trace levels of nitrite and

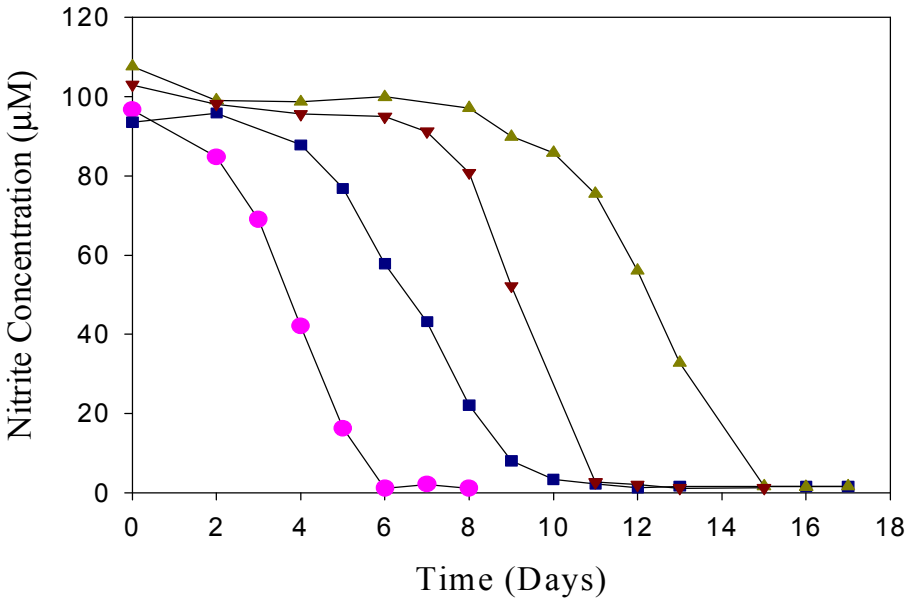


Figure 5.4. Microcosms showing disappearance of nitrite from the soil sample OP1 from the column sections (♦) 0 – 2.5 cm (■) 2.5- 5 cm(▼) 5-7.5 cm (▲) 7.5 – 10 cm

nitrate were found to be present in the soils obtained from the column upon dissection).

Batch tests constructed from soil OP1 confirmed the presence of 2,4-DNT degraders and adsorption and desorption studies showed that 2,4-DNT will not strongly associate with soil OP1. Combined microcosm, sorption and column studies suggest that biodegradation is a likely process responsible for removal of 2,4-DNT from the soil columns. A likely reason for the low levels of nitrite in the soil column could be the oxidative metabolism to nitrate.

Thus the soils were screened for the presence of nitrite oxidizers. The soils from all the four sections of the column showed the presence of nitrite oxidizers with nitrite being oxidized at slightly different rates (Figure 5.4). The top most section of the column (0-2.5 cm) oxidized nitrite within a week and other sections took about two weeks to remove all nitrite. The section (0-2.5 cm) which showed the fastest rate of removal of nitrite was transferred to a fresh flask and the culture was monitored for evolution of nitrate. Stoichiometric release of nitrate was observed in the batch culture which further confirmed the presence of nitrite oxidizers (Figure 5.5). It is speculated that the absence of nitrate from the column effluent can be attributed to nitrate being utilized as a nitrogen/energy source by other bacteria present in the soil.

These results indicate that nitrite oxidizers present in the soils samples may have been responsible for the oxidation of nitrite, explaining the difference in observed vs. expected nitrite based on the influent 2,4-DNT concentration. Thus the continuous observation of low concentrations of nitrite in the 2,4-DNT column effluent and absence of nitrite coupled with the presence of a reduced form of 2,6-DNT indicates that 2,4-DNT, but not 2,6-DNT was biodegraded in the soil columns. The use of simulated

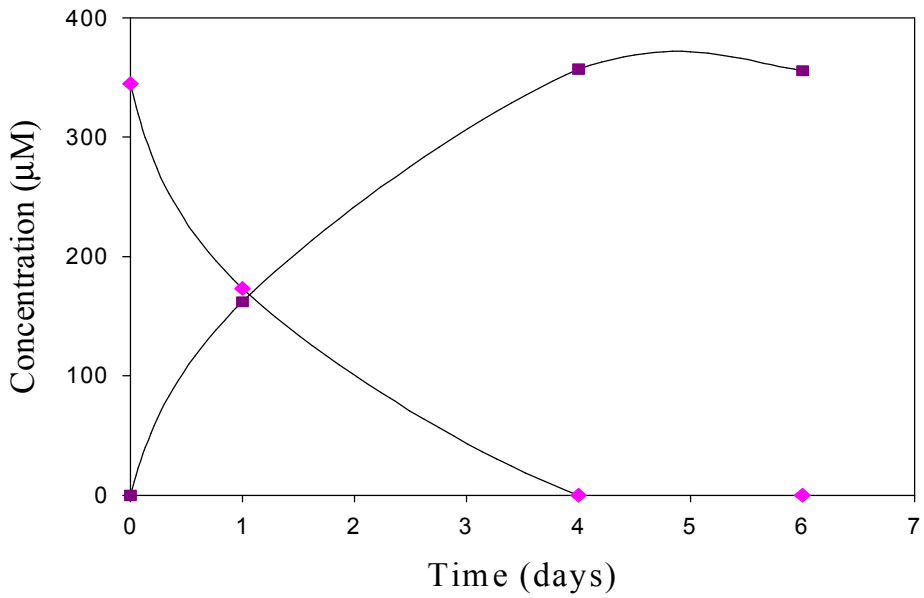


Figure 5.5. Batch culture showing stoichiometric release of nitrate by the nitrite oxidizers obtained from Soil Sample OP1 (♦) nitrite disappearance (■) nitrate production.

rainwater as influent with no nutrient amendments suggests that nutrient limitation is not limiting the biodegradation of DNT at the site. These conclusions are supported by the microcosm studies which showed the presence of 2,4-DNT degrading strains and absence of 2,6-DNT degrading strains. 2,6-DNT contamination is not widespread at the site and is of much less concern than 2,4-DNT (Table 5.1).

These result of this study which showed less than stoichiometric production of nitrite were not consistent with the previous studies involving high levels of DNT which have shown stoichiometric production which has been considered as an indicator of 2,4-DNT degradation [21]. Based on this, it is hypothesized that high levels and not low levels of DNT might be inhibiting the nitrite oxidizers which requires further study.

5.7. Chemostat Study

Seasonal temperature fluctuations of surface soils represent a factor that may strongly influence the rate or extent of DNT biodegradation (at BAAP or at other sites). In a CSTR with a reactor volume (860 ml) and retention time (2.5 days), mineral medium [22] spiked with 2,4-DNT (250 μM) was fed. In this study the operating temperature was reduced stepwise (22°C, 15°C, 10°C, 7.5°C, 4°C) (Figure 5.5). Interestingly, temperature did not have a dramatic impact on 2,4-DNT degradation at steady state and the removal remained high. The effluent concentration at 22°C and 15°C showed 98 ± 0.1 % of DNT removal at steady state whereas at 10°C and 7.5°C, 94% of the DNT removal was observed. The bacteria were being maintained at 22°C 2,4-DNT (250 μM) in a batch culture; hence no lag phase was expected at that temperature. No lag phase or decrease in substrate removal was observed when the temperature of the chemostat was lowered to 15°C. At 10°C initially the DNT consumption decreased to as low as 75 % but gradually

as the bacteria became acclimated, 94 ± 1.3 % substrate removal was observed at steady state. At 7.5°C and 4°C, 94 ± 0.5 % substrate removal was observed which was equal to that observed at 10°C with no lag phase observed.

Along with 2,4-DNT effluent nitrite was also measured. The nitrite concentration at 22°C were found to be about 4.5 times lower than the expected stoichiometric concentration which is 1.63 moles of nitrite evolved for every mole of DNT being degraded [30]. At this temperature the observed concentrations were approximately 80 μM compared to the expected stoichiometric concentrations of 390 μM. The OP1 mixed culture that was used for the experiment also contained the nitrite oxidizing bacteria as discussed above which we believe accounts for lower concentrations of nitrite in the effluent. Even though the lowering of temperature from 22°C to 15°C didn't have any effect on 2,4-DNT degrading bacteria, it had an observable effect on the nitrite oxidizers. The effluent nitrite detected at 15°C increased to 190 μM compared to 80 μM detected at 22°C but still were half of the expected stoichiometric concentration. Further decreasing the temperature to 10°C ceased the nitrite oxidation. Similar observation was made at 7.5°C and 4°C.

Considering these reactor conditions with a hydraulic retention time of 2.5 days, lowering the temperature did not have a large effect on the 2,4-DNT mass removal via biodegradation but had a large effect on nitrite oxidizers.

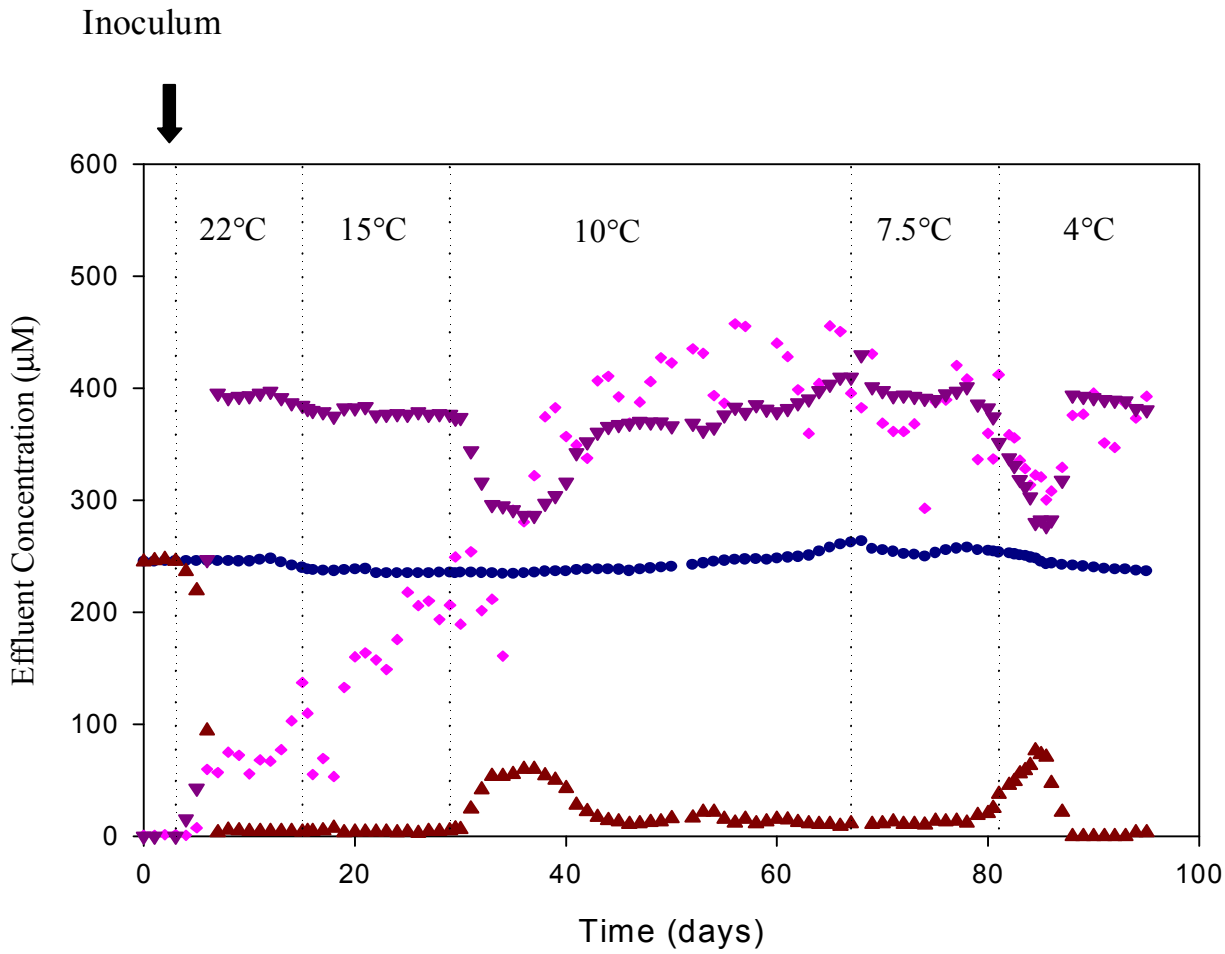


Figure 5.6. OP1 Chemostat effluent concentration vs. time. (-) represents 2,4-DNT concentrations accounting for dilution and no degradation; (▲) represents observed 2,4-DNT effluent concentrations including degradation (●) represents the expected nitrite concentration according to stoichiometry i.e. 1.63 moles of NO_2^- for 1 mole of DNT (♦) represents no nitrite used by bacteria as nitrogen and both the nitro groups on DNT released upon degradation (♦) represents observed nitrite effluent concentrations.

CHAPTER 6

CONCLUSIONS

The microcosm studies demonstrated the presence of bacteria at the Settling Pond and Spoils Disposal areas that are capable of degradation of 2,4-DNT, and thus provide evidence of the presence of the appropriate microbiology at all three sites for natural attenuation to occur. In contrast, the BAAP soils were not found to be source of microbes capable of metabolizing 2,6-DNT.

The tendency of DNT to sorb to the soil and thus become unavailable to the bacteria was studied. All Settling Pond and Spoils Disposal soils examined (OP1, OP4 and SSB) had the propensity to sorb DNT, however, depending on the soil type and properties, the soils did so at different capacities. Soil SSB had the greatest capacity which was evident in the relatively large experimentally determined K_F values (K_F for 2,4-DNT = 342.5 L/kg; K_F for 2,6-DNT = 278.3 L/kg). In contrast, soils OP1 and OP4 were observed to have lower K_F values, thus less soil associated DNT. Desorption data showed similar trends with higher clay and organic content soils, SSB in particular, required more desorption steps than OP1 and OP4 before undetectable levels of DNT was desorbed. These observations were supported by additional desorption studies examining the background 2,4-DNT levels associated with SSB upon arrival from BAAP. Despite having an appreciable 2,4-DNT concentration associated with the aged contaminated soil SSB (30 mg/kg of 2,4-DNT), after five days of suspended conditions in water, aqueous phase concentrations remained below detection limits.

Thus the sorption studies showed that DNT will adsorb reversibly and become bioavailable, desorbing at different rates depending on the soil properties.

Effluent data from the OP1 soil column study suggests that 2,4-DNT was being degraded within the column, while 2,6-DNT which reached an equilibrium effluent concentration of 3.6 mg/L after three weeks of operation. Throughout 123 days of column operation, 2,4-DNT was never detected in the effluent and no 2,4-DNT was found in the column soils upon dissection at the conclusion of the study. However, nitrite was observed, though much lower than expected (due to the presence of nitrite oxidizers) as compared to 2,6-DNT column which showed no nitrite. These findings, coupled with the confirmation of presence of nitrite oxidizers and low K_F value of OP1 suggest that biodegradation is responsible for loss of 2,4-DNT in the soil column study.

The use of stimulated rainwater as influent with no nutrient amendments suggests that a lack of nutrients is not limiting the biodegradation of low concentrations 2,4-DNT in the soils tested.

Fortner *et. al.* [21] studied biodegradation of DNT in highly contaminated vadose zone soils in soil column with external aeration to prevent the inhibition of DNT biodegradation due to lack of oxygen. The biodegradation of DNT observed without any external aeration in this study suggests that availability of oxygen is not limiting the biodegradation in the soils tested.

The chemostat studies were conducted to study the effect seasonal temperature fluctuations at this particular site on the biodegradation of DNT. Considering the reactor conditions, lowering the temperature from 22°C to 4°C did not have an effect on the DNT mass removal via biodegradation with a hydraulic retention time of 2.5 days. At these temperatures, 2,4-DNT degradation rates remained fairly constant. The effluent concentration at 22°C and 15°C showed 98% of DNT removal at steady state whereas at

10°C and 7.5°C, 96% of the DNT removal was observed. However, lowering the temperature had an observable effect on the nitrite oxidizers. The nitrite oxidizers were actively oxidizing nitrite at 22°C, the activity decreased by a factor of two at 15°C an ceased at temperatures 10°C and lower. Thus, lowering the temperature did not have any observable effect on the DNT degraders and it will not withhold the ongoing natural attenuation process occurring at the site at the site at temperatures.

CHAPTER 7

ENGINEERING SIGNIFICANCE

Natural attenuation is an accepted and often cost-effective alternative to other intrusive options for soil remediation such as ex-situ slurry reactors, composting, and incineration. Also, natural attenuation offers ecological improvement, simplicity in concept, and aesthetic advantages with strong public acceptance. Based on converging lines of evidence presented here, natural attenuation should be strongly considered as a remedial option for BAAP and similar sites where low concentrations of DNT isomers are present as contaminants.

Nitrite is taken as a line of evidence that biodegradation of DNT is an active processes at the site. This research showed that nitrite measurement should not be always taken as a conclusive indicator of DNT degradation. The on-site remedial personnel should take into consideration the fact that absence of nitrite does not necessarily mean absence of DNT biodegradation at the site.

The chemostat studies conducted to study the effect seasonal temperature fluctuations on the biological degradation of DNT showed that the nitrite observed at temperature 22°C was lower than the nitrite observed at 15°C which in turn was lower than the nitrite observed at temperatures 10°C and lower, because of the presence of nitrite oxidizers. Thus while monitoring natural attenuation during different seasons it should be kept in mind that none or lower nitrite concentrations at higher temperatures does not indicate none or lower DNT degradation. Similarly, higher nitrite concentrations at lower temperature compared to nitrite concentrations observed at higher temperatures

does not indicate faster or higher DNT degradation but might suggest lower or no activity of nitrite oxidizers. Thus engineers at the site should be aware of the fact that the fluctuation of nitrite measurement does not necessarily indicate fluctuation in DNT degradation.

CHAPTER 8

FUTURE WORK

Based on the results observed in this research it is proposed that a pilot scale field study should be conducted to understand biodegradation together with other processes such as sorption, dispersion and, diffusion in natural attenuation of DNT on a larger scale before implementing monitored natural attenuation (MNA) as a remediation strategy at BAAP or other sites. It is also proposed that microbial ecology at other sites and cultures should also be studied to understand whether the results observed in this study are site specific or not.

Nitrite evolution is generally taken as an indicator of DNT degradation while monitoring natural attenuation. Stoichiometric production of nitrite has been observed while working with high levels of DNT. However, the same result was not observed while working with low levels of DNT. As observed in the soil columns nitrite was oxidized to nitrate which was then expected to be removed by the other microbes which used nitrate as a nitrogen/energy source. Thus the compound used as an indicator of biodegradation of DNT and thereby used for monitoring of natural attenuation was absent. It is hypothesized based on the discrepancy observed in the production of nitrite at high and low concentration of DNT and the ubiquitous presence of nitrite oxidizers, that DNT at certain concentrations might be inhibiting the nitrite oxidizers. Even if nitrate was the endpoint, it is very mobile and will be easily lost through leaching. Also, there could be other sources of nitrate such as ammonia converted to nitrate in the nitrogen cycle and thus it might make it difficult to say for sure whether natural attenuation is occurring or not [50]. Probably the nitrite oxidizers should be isolated from these soils

and their physiology should be evaluated. It is also proposed that effect of concentration of DNT on nitrite oxidizers should be studied further to fully understand the effects of high levels of DNT concentrations on the nitrifying bacteria. It would give the on-site remedial personnel a better way of evaluating the biodegradation of DNT as a natural attenuation process.

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